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MARINE AND FRESH-WATER HARMFUL ALGAE

PROCEEDINGS OF THE 16TH INTERNATIONAL CONFERENCE ON HARMFUL ALGAE

27 – 31 October 2014 • Wellington, New Zealand

Editor: A. Lincoln MacKenzie

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EDITOR

A. Lincoln MacKenzie

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Preface

The 16th International conference on Harmful Algae was held at the Michael Fowler Centre in Wellington, New Zealand, over 5 days between the 27th and 31st October 2014. The conference was hosted by the Cawthron Institute, Nelson, New Zealand's largest independent environmental science research organisation.

The theme of the conference was ***Advancement Through Shared Science*** in recognition of the multidisciplinary nature of the field and the important role international collaboration has played in understanding HAB phenomena and managing and mitigating their effects. The conference was opened by Mr Scott Gallacher, the Deputy Director General of the New Zealand Ministry for Primary Industries and Professor Charles Eason, Chief executive of the Cawthron Institute.

The conference was attended by 385 delegates from 34 countries. There were 196 oral presentations, each of 15 minutes duration with 5 minutes question time, and 150 poster presentations. The conference had good representation from both the Freshwater and Marine sectors of the HAB research field. Because of the large number of talks it was necessary to have three parallel sessions throughout the conference. However the venues were in close proximity to each other and timely exchanges between them went well due largely to the accurate time keeping by the session chairs, to whom the organisers were most grateful. In an innovation for this conference series, there were two additional sessions (total of 19 papers) of 5 minute speed talks in which the presenters gave a series of succinct, well prepared and interesting talks on a range of topics.

The oral presentations were divided into the following sessions:

- Taxonomy, systematics and genomics
- HAB life cycles
- HABs in a changing world
- Freshwater HAB Biology and Ecology
- Economic effects of HABs
- HAB molecular taxonomy and ecology
- HAB transcriptomics
- HAB parasites
- HAB dynamics and modelling
- Heterotrophy and mixotrophy
- Algal toxins
- HAB mitigation
- Toxicology
- Health effects of HABs
- Surveillance and management
- Ciguatera
- HAB technologies
- HAB Ecology and physiology
- HABs and Bivalves
- Freshwater benthic HABs
- Dinoflagellate cysts
- Ostreopsis
- Ichthyotoxic HABs

On the first and last day there were Keynote addresses by Professors Øjvind Moestrup (University of Copenhagen) and Hans Paerl (UNC-Chapel Hill Institute of Marine Sciences). Professor Moestrup discussed the species concept and current issues in the area of naming and identification of harmful algae, brought about through the use of molecular methods that do not always coincide with traditional classifications based on morphological characteristics. Professor Paerl discussed how CyanoHABs are regulated by nutrients (both N & P), light, temperature, stratification, water residence time and biotic interactions. His main thesis was that future rising temperatures and greater hydrologic variability will exacerbate problems with CyanoHABs and nutrient controls may need to be more aggressively pursued in response to global climate change. The seven Plenary speakers provided extended talks that introduced each major topic area.

These proceedings contain 9 papers by all the Keynote and Plenary speakers and 54 papers from other contributors organised into seven chapters covering broad topic areas (Algal Toxins and Toxicology, Freshwater HAB Biology and Ecology, Marine HAB Biology and Ecology, Ciguatera, HAB Technologies, HAB Transcriptomics, Surveillance and Management). Thanks are due to the referees who peer reviewed submitted manuscripts within their area of expertise.

A key component of the success of the conference was the unflappable professionalism of the conference organisers from ***Conferences and Events***. Special thanks are due to Michelle, Kane, Amy Abel and Francie Gaiger who dealt with the nuts and bolts of registration and abstract submissions. They fielded many enquiries from attendees and ensured that the handbook and abstracts were well presented, that the conference ran smoothly and delegates were well fed and watered. Thanks also to our sponsors who enabled us able to provide a good standard of hospitality to the delegates.

The organising committee are grateful to our colleagues who made the long trip to New Zealand to attend the conference and contributed the large number of excellent presentations that made it so worthwhile and successful.

Hei konā mai i roto i ngā mihi

(Goodbye and thank you)

A. Lincoln MacKenzie (PhD) Convener

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Keynote and Plenary Presentations

On identification of harmful algae and the species concepts

Øjvind Moestrup

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Abstract

During the history of science, development and application of new techniques have often impacted our perceptions of the total number of plant and animal species on the globe, one reason being changes in the species concept(s) used. We are presently going through a period during which molecular data are being published on harmful algae from all parts of the world and, perhaps not surprising, our concepts of species using this newest technology, indeed our concepts of classification at all taxonomic levels, are changing once again. Generally taxonomic concepts are becoming more narrow (taxonomic inflation), more rarely, presently recognized species are merged into fewer species. Monophyly at all levels is the mantra, usually based on a combination of morphological, biochemical and morphological information, or occasionally on one of these types of data only. Thus, what used to be a single species, *Gambierdiscus toxicus* has now been split into a dozen or so species, very recently even into two genera, and what used to be the class Chrysophyceae has now been split into 6 or 7 classes, etc. This has an impact not only on scientists but also on people using the taxonomy created by these scientists. I discuss some of the problems associated with naming and identification of harmful algae and how to meet the challenges facing scientists and monitoring personnel, who aim to identify and enumerate harmful algae for monitoring purposes. At first, I provide a short presentation of the IOC UNESCO courses on harmful microalgae, which began in 1995 in Copenhagen, aimed at identification of the algae, and of the IOC UNESCO Taxonomic List of Harmful Microalgae.

Keywords: species concepts, HAB monitoring

E-mail received during preparation of this manuscript:

"After publishing a new concept of *Alexandrium tamarense* complex, many Japanese scientists, who are working in monitoring HAB events around aquaculture areas are totally confused in particular disappearing the species name of *A. catenella*. Is there any discussion and conclusion on this issue?" (e-mail from a colleague in Japan, 11.5.2015)

The taxonomic courses in Copenhagen and elsewhere

Since its establishment in 1995, the IOC Science and Communication Centre on Harmful Algae at the University of Copenhagen has arranged or participated in international courses on harmful algae both in Denmark and abroad, and the courses are still ongoing. The total number of participants in the courses is now close to 1000, from ca 60 countries. A major emphasis on the courses has been taxonomy and how to identify different species of harmful algae. The taxonomic groups of algae are discussed, and as many as possible of the harmful species are demonstrated and discussed using light microscopy and cultures supplied by the Scandinavian Culture Collection of Algae and Protozoa (SCCAP). After a few years, an initial internet e-learning was incorporated, in which the participants were taught basic information about the algae. This was followed by a (shortened) course in Copenhagen or elsewhere. Following a recommendation from Phil Busby, New Zealand, at an IOC UNESCO meeting in Paris, some courses were further developed to end with a written exam, which participants had to pass satisfactorily to obtain the course certificate. Participation in the courses could then be used as a guarantee that participants had obtained knowledge of harmful algae.

The taxonomic database

At around the same time, a database was established on request by the IOC Intergovernmental Panel on HABs, listing the toxic marine microalgae known at the time. The objective was to establish a common database, available on the internet, on which users could check which species were known to be harmful, and their harmful potential. The aim was also to ensure that a list of correct names and author citations of each species was readily available. An international editorial committee was set up, with expertise covering the different taxonomic groups

of algae. The list was initially available on the homepage of Copenhagen University but subsequently the list became integrated into the World Register of Marine Species (WoRMS). The main WoRMS list comprises all marine species of plants and animals, and presently runs into ca 220,000 species. The IOC UNESCO Taxonomic Reference List of Harmful Micro Algae retained some individuality after its engulfment by WoRMS, and can be accessed as www.marinespecies.org/hab/. Very recently, the WoRMS list associated with the AlgaeBase list in Galway, Ireland, the goal of which is to list all the world's algae, freshwater, terrestrial or marine. The names used in WoRMS now agree with the names used in AlgaeBase, and therefore also with the IOC-UNESCO Taxonomic Reference List of Harmful Micro Algae. If name changes occur, they will now be inserted at the same time in AlgaeBase, the general WoRMS list and the IOC Reference List.

Species diversity and how to identify species

Over the years, changes have taken place in both generic and species names of harmful algae. Name changes are often a result of new technical developments, thus major changes took place after the introduction of transmission electron microscopy (TEM), followed by scanning electron microscopy (SEM). Using TEM it became clear from the 1950s onwards that species diversity in the haptophyte genus *Chrysochromulina* was much higher than thought at the time, the species differing particularly in the complement of organic scales that form a periplast on the cell. The scales can in many cases not be visualized even in the best light microscope. Description of new species has presently (2015) slowed down somewhat, but can be expected to increase again since numerous undescribed species occur, even in the well-studied waters of Scandinavia. Conventional monitoring of these species by light microscopy is no longer possible, and even TEM can fail to provide proper names as the organic scales on the cell readily drop off the cell in many of the fixatives used for monitoring purposes. Using light microscopy many haptophyte species can presently be assigned to the group Haptophyta only.

Introduction of SEM had an impact particularly on the taxonomy of the larger species such as diatoms and dinoflagellates, the resolution being insufficient to allow details of the organic scales of haptophytes and other scale-bearing organisms to be studied. When a species of the diatom genus *Pseudo-nitzschia* was identified in 1987 as the cause of domoic acid contamination of shellfish, it was identified by light- and electron microscopy. This first-found toxic species, *P. multiseries*, is morphologically very similar to *P. pungens*, which is usually non-toxic, and the two species can only be reliably identified by electron microscopy, preferably by TEM or SEM, which allows study of the poroid pattern that distinguishes the two species. TEM is often used to identify species of *Pseudo-nitzschia*, but following the description of new species in the *delicatissima* and *pseudodelicatissima* complexes, differences between some of the new species are very small and difficult to see in the TEM.

For monitoring by light microscopy, identification of live or fixed whole cells or colonies of *Pseudo-nitzschia* to species level is, with few exceptions, not possible. Cleaning of the cells and studies of the frustules in a high-resolution light microscope increases the number of species that can be identified by light microcopy. TEM or SEM increases the number even further.

However, microscopy, light or electron, is no longer sufficient to identify all species of *Pseudo-nitzschia* to species level. New (cryptic) species have been described (Quijano-Scheggia *et al*. 2009), which can be identified to species level only by molecular sequencing.

Molecular sequencing, yet another new technique, which began to be employed in the late 1980s and more regularly from the 1990s, is presently having an increasing impact on our ideas of classification of algae into species, genera, families, etc, in fact on all levels of classification. This has sometimes resulted in a clash with conclusions reached by using other methods, and we are presently experiencing a period of transition, the end result of which can only be surmised. Some of the molecular data obtained are used to split species and genera into smaller units, others to merge previously recognized species, and others again to redefine previously recognized genera and species. This can have considerable impact on how to identify species and on monitoring. Before discussing how these problems may be handled and solved, I will provide examples of taxonomic problems facing us at the present time. Also on which techniques are now required to identify the individual species of harmful algae.

Cyanobacteria

Several studies on harmful cyanobacteria contain molecular data, and some of the most

controversial are probably those on *Microcystis*. Otsuka *et al*. (1998, 1999, 2001), based on 16S rDNA and ITS, formally suggested merging a number of the well-known morphospecies of *Microcystis*: *M. aeruginosa*, *M. ichthyoblabe*, *M. novacekii*, *M. wesenbergii* and *M. viridis*. Several of these taxa, which are distributed worldwide, are well known and readily distinguishable in
the light microscope. In culture they sometimes change morphology and identification to species or even generic level becomes difficult or impossible. The new proposals recall the series of studies by Drouet and Daily in a number of books 1956-1981, on a very large number of collections of cyanobacteria, in which the authors proposed to reduce the many hundred described species of cyanobacteria described at the time to only ca 60 (summarized by Drouet 1981). The new system was used for a short time but then slowly disappeared, I assume because the species concept was far too broad.
In contrast to the conclusions mentioned above, Piccin-Santos *et al.* (2014), based on sequencing of the non-coding phycocyanin-IGS region, found the 17 strains of *Microcystis wesenbergii* examined to form a monophyletic group. To base the taxonomy of *Microcystis* on molecular data is therefore not straight forward, there appears to be no alternative at the moment to conventional monitoring by light microscopy

Raphidophyceae
Demura *et al*. (2009) studied three taxa of fish-killing raphidophyceans, *Chattonella antiqua*, *C. marina* and *C. ovata*, by combining cell size and cell morphology data with molecular data from ITS, rbcL, COI and selected microsatellite regions. The authors concluded that the characters overlapped to such an extent that they should be considered to belong to the same species. On the other hand, based on the morphological and molecular data, the many (104) strains formed three groups, roughly corresponding to the three morphospecies. The authors considered the genetic difference between the three groups insufficient to justify distinction at the species level, but suggested to consider them varieties of a single species, *C. marina*. In culture, individual cells of the three taxa could not always be distinguished because of variability in morphology.

Diatoms
The number of diatoms on the globe is being debated, with the number of described species expected to rise from the ca 10 000 species or so presently described, to ten times as many or perhaps even more. A considerable increase has taken place also in the number of harmful diatoms. The number of species in the genus *Pseudo-nitzschia* as a whole – including both toxic, sometimes toxic and non-toxic species - has approximately doubled since diatoms were first discovered to produce toxins in 1987. Very few of the species can be identified from live or fixed material, but cleaned material of most species can be identified by electron microcopy. However, as mentioned above, very recently cryptic species have been described which are morphologically identical or nearly so to other species, even in the transmission electron microscope, but differ in their molecular fingerprint. When such cryptic species are shown to be toxic, molecular sequencing will be required to identify them. A further concern is presently species of *Nitzschia*, two of which have now been found to produce domoic acid, one species from Vietnam (Lundholm & Moestrup 2000) and one from Tunisia (Smida *et al.* 2014). Considering that species of *Nitzschia* are notoriously difficult to identify (nearly 1000 taxa have been described in total from freshwater and marine waters), we may expect future problems with identification when/if more *Nitzschia* are found to be toxic.

Dinoflagellates: Alexandrium
The problems associated with the *Alexandrium tamarense* complex are presently some of the most notorious (cf. the e-mail referred to at the beginning of this article). The tamarense complex until recently comprised three species, defined on morphological characters such as presence or absence of a pore in the first apical plate, the shape of the cell, and the ability of the cells to form colonies. Lilly *et al*. (2007), followed by John *et al.* (2014), used ribosomal sequences (SSU, ITS, LSU) to investigate a large number of clones belonging to the tamarense group collected from many parts of the world. They found that, based on ribosomal data, the clones fell into five groups (clades). John *et al.* (2014) provided a number of reasons for providing each group with a separate species name (ribospecies). The clones of the five clades are closely related, and studies on non-ribosomal genes are urgently needed before these suggestions can be fully evaluated. What makes the situation unusual is a discrepancy between the morpho- and the ribospecies concept: three morphospecies or 5 ribospecies.

If further sequencing, using non-ribosomal genes, corroborate the division of the clones of the *Alexandrium tamarense* complex into the five phylogenetic species suggested, creation of subspecies or varieties within some of the ribospecies will become necessary to retain information about morphotype.

Dinoflagellates: Gambierdiscus and some other dinoflagellates
What used to be a single species of *Gambierdiscus* has recently been split into a dozen species, and most recently these have become separated into two genera, *Gambierdiscus* and *Fukuyoa* (Gomez *et al.* 2015). Each species of the *Gambierdiscus* complex is defined by a set of molecular and morphological characters. Considering, however, that the morphological characters separating the species are small, and that variation occurs in plate patterns and morphology also in nature, molecular methods are likely to be required if identification to species is needed. The same applies to other species which are difficult to identify using light microscopy only, including species of the Pfiesteriaceae, species of *Karenia* and *Karlodinium*, etc. In one genus, the (non-toxic) symbionts of coral, *Symbiodinium*, many strains are morphologically identical (cryptic species) but differ in their molecular signature.

General

Merging or splitting
We obviously need some system of naming to communicate about taxa, whether genera, species or varieties. Splitting into smaller units increases the number of species to such an extent that conventional monitoring by light microcopy becomes difficult or impossible, but splitting may be scientifically sound. Merging of species can be problematic for a different reason. To merge into large units may hide or obscure information we want to communicate. Thus to merge all five species of *Microcystis* mentioned above into a single species, *M. aeruginosa*, may make identification simpler, but it reduces the value of the information provided by the name *M. aeruginosa*. The same applies to the three species of *Chattonella*. To merge them under the same name without variety status hides information (antiqua type, marina type, ovata type?) but retaining them as varieties ensures that information about morphotype is kept. Whether the morphotype is considered a species or a variety appears to be less important, no information is lost.
To merge all clones of the *Alexandrium tamarense* complex into five ribospecies also hides morphological information. Thus Group I comprises all three morphotypes and by using the species name *A. fundyense* for all clones of the group (John *et al.* 2014), no information is given on whether the clones in question are morphologically of the *catenella*, *tamarense* or *fundyense* morphotype.

Is there a single solution in sight?
There are presently some 20 different species concepts. Most are based on the biological species concept, the morphological species concept, or the phylogenetic species concept. The biological concept is occasionally used for harmful algae, but the morphological concept is the norm although the phylogenetic species concept is becoming more common. The increasing use of sequencing and the quest for monophyly questions many of the previous classifications. In many cases definition of species, genera, families, etc is, however, a matter of personal preference. Thus it is well known that freshwater species of *Ceratium* differ very slightly in plate formula from the marine species of the genus, and the morphological difference between the two groups is supported by molecular studies. The genus *Ceratium* is still monophyletic, however, (Gomez *et al*. 2010) and it is a matter of personal preference whether it should be split into subgenera (as in *Chaetoceros*) or into two separate genera, *Ceratium* from freshwater and *Tripos* marine. How to define species and genera has been discussed as long as I can remember, and there seems to be no end in sight. In the early 1990s a very competent colleague said to me that by the year 2000 most essential information on phylogeny and classification has been obtained. He has subsequently changed his wording.
Molecular data have provided very important information about genetic diversity and phylogenetic relationships, and the combination of morphological and molecular data have contributed to solve many taxonomic problems and to give information about areas of ongoing speciation. However, the data have also contributed to increase the difficulties in defining taxonomic units.

Future monitoring
What is the future for monitoring of harmful algae? If the present trend continues, molecular

methods will soon become necessary tools for identification of many species, and for monitoring. A beginning was made in Monterey Bay some years ago, when data on species of harmful algae, including *Pseudo-nitzschia*, *Alexandrium* and *Heterosigma* were collected by moored *in situ* sensors containing relevant molecular probes, and uploaded by satellite to a shore station (Scholin *et al*. 2008; further information in Ryan *et al.* 2011). Cellular domoic acid concentrations were quantified periodically. Such methods are quick, more or less real-time, and the time-consuming delay caused by transportation of samples, sedimentation, identification and counting is avoided. To employ this and other types of sophisticated methods in other parts of the world will require development of molecular probes for all harmful species (see Brosnahan *et al.* 2013 for another method utilizing probes).
It is a goal of the future to agree on a common species concept, if possible, and to harmonize on a global scale the methods used for monitoring. The nine different countries around the Baltic Sea have made a promising start in the HELCOM project (HELCOM 2014).

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Managing Harmful Cyanobacterial Blooms in a World Experiencing Anthropogenic and Climatically-mediated Change

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Abstract

Cyanobacteria have a long evolutionary history, which has allowed them to adapt to geochemical and climatic changes and more recent anthropogenic modifications of aquatic environments, including nutrient over-enrichment and altered hydrologic conditions. Bloom-forming harmful cyanobacterial genera (CyanoHABs) can be harmful from environmental, organismal and human health perspectives by outcompeting beneficial phytoplankton, creating low oxygen conditions and producing cyanotoxins. Because CyanoHABs exhibit optimal growth rates at relatively high water temperatures, global warming plays a role in their expansion and persistence. CyanoHABs are regulated by the combined effects of nutrient (nitrogen; N and phosphorus; P) supplies, light, temperature, vertical stratification, water residence times, and biotic interactions. Control strategies should generally be focused on reducing *both* N and P inputs. Physical, chemical and biological manipulations can reduce CyanoHABs; however, these strategies are restricted to small systems, and are prone to secondary effects, including release of cyanotoxins, disruption of habitats and trophic changes. Strategies should be adaptive to climatic change for long-term control of CyanoHABs. Rising temperatures and greater hydrologic variability will increase growth rates and alter critical nutrient thresholds for CyanoHABs; therefore nutrient input controls may need to be more aggressively pursued in response to climatic changes globally.

Keywords: Harmful cyanobacteria, nitrogen, phosphorus, water quality management, mitigation, climate change.

Introduction

Blue-green algae or cyanobacteria are among the most ancient phototrophs on Earth and their "invention" of conducting oxygenic photosynthesis has been the single most important biologically-mediated "event" shaping the modern day oxic biosphere (Schopf 2000). Diverse cyanobacterial communities inhabit every corner of the Earth, ranging from the oligotrophic open ocean to barren deserts, alpine and polar regions (Potts and Whitton 2000). A major attribute of the cyanobacteria is their ability to survive and thrive in physically and chemically extreme environments. Presumably, this capability stems from their long evolutionary history (~ 3 bya) (Schopf 2000), during which they experienced virtually all climatic changes and extremes that have been recorded in geological history. Adaptations to these extremes are widespread among cyanobacterial taxa, including the formation of heat and desiccation-tolerant resting cells or akinetes, the presence of photoprotective and desiccation-resistant sheaths and capsules, a wide array of photoprotective (including UV protective) cellular pigments, the ability to glide and (in aqueous environments) rapidly adjust and optimize their position in the water column in response to irradiance and nutrient gradients. They have also developed a wide array of physiological adaptations to variations in nutritional conditions and constraints, including the ability to "fix" atmospheric nitrogen (N_2), sequester (by chelation) iron, store phosphorus (P), nitrogen (N) and other essential nutrients, and produce various metabolites that enhance their ability to counter potentially adverse conditions in their immediate environment, including photooxidation (Potts and Whitton 2000; Paerl and Otten 2013), and yet to be discovered protective and adaptive functions. Lastly, cyanobacteria have formed a diverse suite of mutualistic and symbiotic associations with prokaryotic and eukaryotic microbes, plants and animals, that are designed to ensure their (as well as their partners') survival in

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environments too challenging and hostile to allow for individual partners to survive in.

Physiologically and ecologically, cyanobacteria are bacteria as opposed to algae, a very important distinction when we consider growth and reproductive dynamics in the context of climate change. As bacteria, cyanobacteria exhibit temperature-growth relationships that are much more like *E. coli* than plants, where growth optima are at >30°C , as opposed to typical eukaryotic algal taxa, among which temperature optima for growth are typically more in the range of 15-25°C. Cyanobacteria are also metabolically quite flexible, being able to grow optimally at very high nutrient supply rates, while also being able to subsist on very low supplies of key nutrients. These adaptations play key roles in a regime of changing climatic conditions.

There is little doubt that the Earth is experiencing climatic changes and extremes (IPCC 2012). To what extent these are attributable to long-term geophysical changes (like the Earth changing its orbit, tilt, etc.) vs. human-induced changes, such as enrichment of atmospheric greenhouse gases (CO_2, methane, N-gases), is debatable. However, one thing is certain; the climatic variability (and extremes) we are experiencing is having a profound effect on the activities, distributions, and survival of many plant and animal species. This variability and extremeness represent opportunities to cyanobacteria, in part because "they've seen it all", and in part because many of the changes that we have been able to document over the past several decades benefit their abilities to compete and establish dominance in aquatic environments.

Climatic changes promoting CyanoHAB dominance

Warming: Global warming is one of the most pervasive symptoms of climate change. Virtually everywhere the globe is warming, but the symptoms are most obvious in temperate and high latitude regions (IPCC 2012). In high latitude regions, lakes, reservoirs and rivers have shown warming of surface waters, leading to earlier "ice out" and later "ice on" periods and stronger vertical temperature stratification (Paul 2008; Paerl and Huisman 2009). This has extended the periodicity and range of cyanobacterial species. Evidence for this exists in lakes in northern Europe and North America (Padisak 1997; Weyhenmeyer 2001; Peeters *et al.* 2007; Suikkanen *et al.* 2007; Wiedner *et al.* 2007).

Greater hydrologic variability: Another symptom of climatic changes is the increasing variability and more extremeness in precipitation amounts and patterns. Storm events, including tropical cyclones, nor'easters, and summer thunderstorms, are becoming more extreme, with higher amounts and intensities of rainfall. Conversely, droughts are becoming more severe and protracted. These events are leading to large changes in hydrologic variability, i.e., wetter wet periods and drier dry periods. This has led to more episodic discharge periods in which large amounts of nutrients are captured in runoff events that can lead to rapid and profound nutrient enrichment of receiving waters. If such events are followed by periods of extended drought in which freshwater flow decreases dramatically and residence time of receiving waters increases, conditions favoring CyanoHABs will improve. This will be further enhanced if it is accompanied by warming (Paerl and Paul 2012). The combination of episodic loads of nutrients (e.g., spring runoff period), followed by a protracted warm (summer), low discharge period will lead to optimal CyanoHAB development.

Higher amounts of freshwater runoff also lead to enhanced vertical density stratification (reduced vertical mixing) in waters containing appreciable amounts of salinity, including estuarine and coastal waters and saline lakes by allowing relatively light freshwater lenses to establish themselves on top of denser saltwater. The resultant enhanced vertical stratification will favor phytoplankton capable of vertical migration to position themselves at physically-chemically optimal depths. CyanoHABs are capable of rapidly altering their buoyancy in response to varying light, temperature and nutrient regimes, and to escape grazers by periodically forming blooms in high irradiance surface waters, which are inhospitable to grazers and eukaryotic taxa (Reynolds 2006). CyanoHAB taxa have photoprotective pigments, enabling them to thrive and persist as surface blooms (Paerl *et al.* 1985). Furthermore, sub-surface dwelling taxa will be shaded by dense surface blooms, leaving them under sub-optimal irradiance; providing an obvious competitive advantage to CyanoHABs.

Climatic effects, synergism, positive feedback loops and CyanoHAB dominance. Thermal and hydrologic, bioactivity and diversity changes accompanying climatic changes operate in a synergistic fashion; that is, their effects are additive in promoting growth, activity and dominance of CyanoHABs. This is accomplished through a series of positive feedback reactions or "loops" that jointly promote bloom development, intensity and maintenance. This scenario is described below: Generally, bloom-forming cyanobacteria are not effectively grazed (due to inedibility and/or toxicity), while non-cyanobacterial phytoplankton taxa are grazed. Nutrient regenerating activities associated with selective grazing tend to favor bloom-forming cyanobacterial genera, at a time of the year (summer months) when these genera are already selectively favored by elevated temperatures. In addition, the enhanced metabolism (O_2 consumption), nutrient cycling and CyanoHAB production associated with grazing will tend to promote bottom water hypoxia, which is further promoted by intensification of vertical temperature stratification. If this hypoxia persists under continued strong vertical stratification and is further "pushed" to anoxia (no detectable DO), then bottom waters may become sulfidic. This condition will favor CyanoHABs, because they are generally unaffected by free sulfide in the water column,

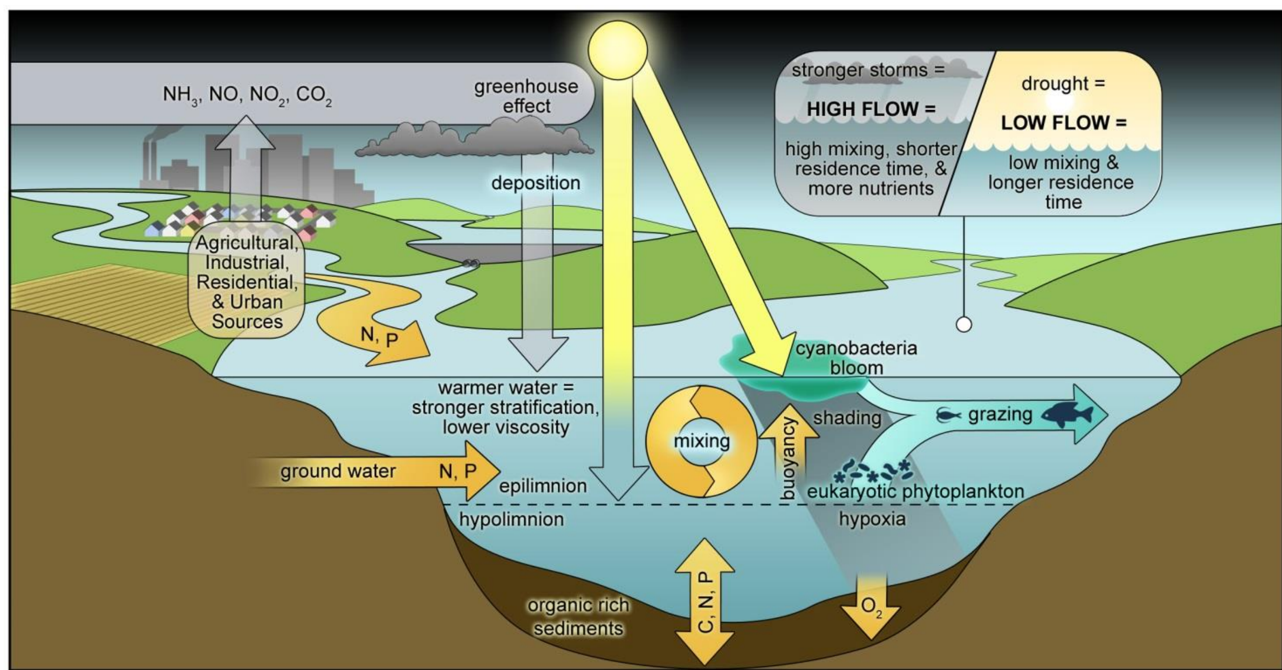

Fig. 1. Impacts of nutrient enrichment, warming, increasing hydrologic variability, and extremeness on physical-chemical and biotic conditions that modulate CyanoHABs in aquatic ecosystems.

which eukaryotic algae cannot tolerate. Hence, CyanoHABs will be able to periodically "dive" (by altering their buoyancy) into nutrient-rich anoxic bottom waters, thereby ensuring a source of nutrients needed to sustain blooms, while eukaryotic algae will be cut off from this nutrient source due to their inability to tolerate sulfide (Fig. 1).

Options for CyanoHAB control in a warmer, more hydrologically-variable world

How do we break the synergism between nutrient over-enrichment and the positive effects of climate change on CyanoHABs? In the short term we can't control weather, let alone the extremeness that appears to be symptomatic of global climate change (IPCC 2012). Therefore, the options for breaking the synergism are focused on managing those environmental factors most closely linked to human activities promoting CyanoHABs. Furthermore, the most practical and effective options should receive priority.

Nutrient input reductions: First and foremost are nutrient input reductions. There is a rich literature that can be used as a guideline for watershed- and ecosystem-specific nutrient input reduction strategies. Phosphorus (P) removal has helped improve water quality in systems impacted by CyanoHABs (Smith and Schindler 2009), but recent studies indicate that these eutrophic systems often exhibit maximum algal growth in response to combined N and P enrichment, or at times only N enrichment (Lewis et al., 2011; Xu *et al.* 2010; 2015; Paerl *et al.* 2014a). These shifts in the freshwater nutrient limitation paradigm, resulting from increasing and uncontrolled N inputs from urbanization and expanding agriculture production, have important management implications. These worldwide changes coincide with increases in freshwater CyanoHABs (Paerl *et al.* 2014a). Severe blooms of toxic, non-N_2 fixing cyanobacteria (e.g., *Microcystis*) continue in nutrient-sensitive systems worldwide, despite improvements from P-focused control (Paerl *et al.* 2014a,b). Because these taxa cannot fix atmospheric N_2, they require combined N sources (ammonium, dissolved organic N, oxidized N) to support growth. Large increases in non-point watershed and atmospheric N inputs fill this requirement. Overall, non-point source loadings of both N and P, including discharge of agricultural fertilizer and waste, urban stormwater runoff, and groundwater inputs, should be prioritized for reductions, because they are often the dominant external nutrient sources (US EPA 2011). Most successful nutrient reduction strategies have targeted both non-point and point sources. The former is often most attractive to target because it can be traced to well-defined sources, while the latter more diffuse, yet quantitatively-important sources often require comprehensive basin-wide management strategies that are difficult to institute and enforce.

Physical controls: Manipulating physical factors, including altering turbulence, vertical mixing and hydrologic flushing can affect CyanoHAB development and dominance. Vertical thermal or salinity stratification and long water residence (or short flushing) times tend to favor CyanoHABs over eukaryotic phytoplankton. Therefore altering the above physical regimes can modulate CyanoHABs (Fig. 1). Vertical mixing devices, bubblers and other means of destratification have proven effective in controlling CyanoHABs in small lakes and reservoirs (c.f. Visser *et al.* 1996). In addition, ultrasonic emission devices aimed at disrupting surface blooms have also been deployed. These techniques are generally not applicable in large lake, estuarine and coastal waters, because they are ineffective in exerting their forces over such large areas and volumes.

Increasing the flushing rates, and thereby decreasing water residence time (or water age), can be effective by reducing the time for cyanobacterial bloom development (c.f., Elliott 2010; Paerl and Paul 2012). While this approach can suppress CyanoHABs, it requires large amounts of low-nutrient freshwater supplies for flushing; a commodity generally not available, especially in drought-stricken regions.

Non-nutrient chemical controls: Chemical treatments have been used to control CyanoHABs. These include the applications of algaecides, the most common of which is copper sulfate. Copper sulfate is effective, but it can be toxic to a wide variety of plant and animal species and its residue in the sediments is problematic as a legacy pollutant. Hydrogen peroxide has also been shown to be an effective algaecide (Matthijs *et al.* 2012). It appears

selective for cyanobacteria (vs. eukaryotic algae and higher plants), and poses no serious long-term pollution problem. Both treatments can cause endotoxins (e.g., microcystins) to be released into the ambient waters upon death of the organisms. Peroxide treatment is more desirable than copper sulfate, especially in drinking water supply systems, because light-stimulated oxidation by peroxide breaks down microcystins into peptide residues (Matthijs *et al.* 2012).

Chemical precipitation of P has also been used. Precipitants include lime ($Ca(OH)_2$), which reacts with the natural alkalinity in waters to produce calcium carbonate. Hydrated aluminum sulfate (alum), ferric chloride or sulfate, and ferrous sulfate are all used widely for P removal in wastewater treatment plants and in small ponds and impoundments (US EPA, 1981). "Phoslock" uses a bentonite clay infused with the rare earth element lanthanum. The lanthanum ions are electrostatically bound to the bentonite, while also strongly binding to phosphate anions. The bound phosphate then settles out of the water column and the thin layer (~1 mm) of Phoslock on the sediment surface forms a barrier to phosphate diffusing out of the sediments. Phoslock has been shown to be reasonably effective in small reservoirs, where it can lead to P-limited conditions that can control CyanoHAB production (Robb *et al.* 2003). However, in larger systems, wind-driven sediment resuspension negates the effectiveness of this and other P precipitating techniques. All precipitation treatments must often be repeated seasonally and annually, hence they are expensive. Lastly, they do not solve P over-enrichment problems, and their effects on internal N cycling are unknown. It is also unknown whether climate change and the associated changes in P input and internal P cycling will influence the efficacy of immobilization methods.

Removing Sediments: Even when external nutrient inputs are reduced, the legacy of eutrophication in sediments can perpetuate high internal nutrient loads that continue to support CyanoHABs. Therefore, removing sediments has also been considered for long-term CyanoHAB control. Sediment removal involves expensive dredging, disturbance of lake bottoms, which can lead to additional nutrient (and potentially toxic substances) release and destruction of benthic flora and fauna. One successful example of dredging for CyanoHAB mitigation is Lake Trummen, Sweden, a small (~ 1 km^2, mean depth 1.6 m) lake that experienced CyanoHAB related water quality degradation in response to domestic sewage and industrial nutrient inputs during the mid-1900's. Suction dredging the upper half meter of sediments during a 2 year period led to highly significant decreases in nutrient concentrations and CyanoHABs (Petersen 1982). The Lake Trummen success can largely be attributed to its small, easily manipulatable size, and the ability to effectively target reductions of external nutrient loads from its small (13 km^2) watershed, following dredging. In other sediment dredging efforts on sections of large lakes, beneficial results have not been achieved, largely because the legacy of nutrient contamination of sediments is far greater than can be removed. Furthermore, sediments removed from a water body must be exported and deposited out of the drainage basin, in order to avoid sediment-associated nutrients from leaching back into the system.

Biological controls: Biological controls include a number of approaches to change the aquatic food web to increase grazing pressure on cyanobacteria or to reduce recycling of nutrients. Biomanipulation approaches can include introducing fish and benthic filter feeders capable of consuming cyanobacteria, or introduction of lytic bacteria and viruses. The most common biomanipulation approaches are intended to increase the abundance of herbivorous zooplankton by removing zooplanktivorous fish or introducing piscivorous fish. Alternatively, removal of benthivorous fish can reduce resuspension of nutrients from the bottom sediments. Questions have been raised about the long-term efficacy of curtailing cyanobacterial blooms by increasing grazing pressure, because this may lead to dominance by ungrazable or toxic strains (Paerl and Otten 2013). Biomanipulation is viewed as one component of an integrated approach to water quality management in circumstances in which nutrient reductions alone are insufficient to restore water quality (Paerl and Otten 2013). Otherwise, nutrient management is the most practical, economically feasible, environmentally-friendly, long-term option.

Conclusions

Cyanobacteria are globally distributed and their activities and relative roles in production and nutrient cycling dynamics are controlled by a complex set of environmental variables that are heavily influenced by human and climatic perturbations. Their long evolutionary history has enabled them to structurally and functionally diversify, which in turn has enabled them to adapt to short-term (i.e., diel, seasonal, decadal) and longer term (geological) environmental perturbations and more gradual changes. Because they have experienced major and extreme climatic shifts over these time scales, they are well suited to deal with and take advantage of various climatic changes that we are now experiencing, including warming, altered rainfall patterns and amounts, resultant changes in freshwater runoff, flushing and vertical stratification.

In addition to climatically-driven environmental changes known to influence cyanobacterial growth and dominance, the most significant anthropogenically-influenced factors include; 1) nutrient (especially N and P) enrichment, 2) hydrological changes, including freshwater diversions, the construction of impoundments such as reservoirs, water use for irrigation, drinking, flood control, all of which affect water residence time or flushing rates, 3) biological alterations of aquatic ecosystems, including manipulations of grazers (from zooplankton to fish), and lastly 4) the discharge of xenobiotic compounds e.g., heavy metals, herbicides and pesticides, industrial and domestic chemicals, antibiotics and other synthetic growth regulators, all of which affect phytoplankton community growth and composition.

Effective long term management of CyanoHABs using the above-mentioned controls must take into consideration the ecological and physiological adaptations that certain taxa possess to circumvent some controls. Examples include; 1) the ability of N_2 fixing taxa to exploit N-limited conditions, 2) the ability of certain buoyant taxa to counteract mixing and other means of man-induced destratification aimed at minimizing cyanobacterial dominance, 3) specific mutualistic and symbiotic associations that cyanobacteria have with other microorganisms, which promote "internal" nutrient regeneration and can lead to bloom persistence and duration despite controls.

In an overwhelming number of cases, nutrient input reductions are the most direct, simple, and ecologically/economically feasible CyanoHAB management strategy; this is especially true for ecosystems experiencing effects of climate change, including warming and/or increased hydrologic variability and extremes (Fig. 1). Nutrient input reductions that can decrease cyanobacterial competitive abilities, possibly combined with physical controls (in systems that are amenable to those controls) are often the most effective strategies. Nutrient (specifically N) treatment costs can be prohibitive however, in which case, alternative nutrient removal strategies may prove attractive; including construction of wetlands, cultivation and stimulation of macrophytes, stocking of herbivorous (and specifically cyanobacteria consuming) fish and shellfish species.

Water quality managers will have to accommodate the hydrological and physical-chemical effects of climatic change in their strategies. Given the competitive advantages (over eukaryotic algae) that cyanobacteria enjoy in a more climatically-extreme period we are now experiencing, efforts aimed at control and management of cyanobacteria will need to be flexible enough to incorporate this extremeness. For example, nutrient input reductions aimed at stemming eutrophication and cyanobacterial bloom potentials will need to be carefully gaged and potentially changed to accommodate higher cyanobacterial growth potentials due to warming and increasing bloom potentials due to stronger vertical stratification and positive nutrient cycling feedbacks.

Lastly, without a comprehensive strategy to reduce greenhouse gas emissions, future warming trends and their impacts on aquatic ecosystems will likely only lead to further expansion and dominance of these ecosystems by cyanobacteria.

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A review on improving prediction of cyanobacterial blooms using *Cylindrospermopsis raciborskii* as a case study

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Abstract

Recent studies on cyanobacteria in the laboratory and field have shed new light on physiological mechanisms used to access resources, such as nutrients. These strategies allow them to dominate in environments with low and variable nutrient availability. However, little of this information has been incorporated into predictive algal growth models for harmful species. This review uses a case study of a toxic freshwater cyanobacterial species, *Cylindrospermopsis raciborskii* to highlight new findings on nutrient utilization strategies. Studies have found that the classic models of nutrient utilization oversimplify phosphorus utilization strategies. *C.raciborskii,* as with marine cyanobacteria, is capable of active uptake of phosphorus under low phosphorus conditions, with growth adapted to fluctuating phosphorus availability, as well as using a range of organic phosphorus compounds. *C. raciborskii* is a nitrogen fixer, but atmospheric nitrogen is only utilized below thresholds of dissolved inorganic nitrogen availability. Nitrogen fixation is therefore a strategy to deal with fluctuating nitrogen availability. Incorporation of these findings into algal growth models has the potential to improve their predictive ability. However, there remains a major challenge to incorporate strain variability into our understanding of the breadth of physiological responses to environmental drivers.

Keywords: freshwater phytoplankton, phosphorus, models

Introduction

The cyanobacterium, *Cylindrospermopsis raciborskii* (Wołoszyńska) Seenayya and Subba Raju is a filamentous diazotroph which dominates lakes and water reservoirs in temperate and tropical environments throughout the world. *C. raciborskii* can produce a suite of potentially harmful compounds, although the ability to produce them is not universal. Some Australian strains of *C. raciborskii* produce an alkaloid cytotoxin, cylindrospermopsin (CYN), while some Brazilian strains produce paralytic shellfish poisoning (PSP) toxins (Neilan *et al*., 2003). Conversely there is limited evidence of the northern hemisphere strains producing toxins (O'Neil *et al*. 2012).

There have been a number of recent reviews of the physiology and ecology of this species (O'Neil *et al.* 2012; Sinha *et al.* 2012). These reviews highlight the worldwide dominance of *C. raciborskii* and indicate that it has physiological adaptations which allow it to outcompete other species. However, the competitive advantage of these adaptations is not immediately obvious. For example, *C. raciborskii* prefers low light and stratified conditions, and hence is neutrally buoyant. Therefore, it regulates its position in the water column to avoid excessive light but remain within the surface mixed layer (O'Brien et al. 2009; Kehoe 2010). However, it is in the flexible strategies used to access nitrogen and phosphorus that *C. raciborskii* appears to be gaining a competitive advantage. This paper examines our current knowledge of this, and the implications for predictive modelling.

Phosphorus

Much of our understanding of phosphorus utilization has come from recent studies of non-harmful marine species. These studies have found that our traditional thinking about phosphorus availability and use have been overly simplistic, and phytoplankton have a much wider range of strategies to access P than previously thought (e.g. Dyhrman, 2008; Dyhrman in press).

Recent studies on utilization of phosphorus by *C. raciborskii* have shed light on a range of flexible strategies that may explain it's dominance in a range of freshwater lakes and reservoirs. This species possesses the *Pst* gene cluster, giving it the ability to actively take up dissolved inorganic phosphorus (DIP) under low phosphorus

A. Lincoln MacKenzie [Ed]. *Marine and Freshwater Harmful Algae 2014*. Proceedings of the 16th International Conference on Harmful. Algae. Cawthron Institute, Nelson, New Zealand and the International Society for the Study of Harmful Algae (ISSHA)

conditions (A. Willis, unpubl. data). However, this ability is not unique to *C. raciborskii.* It has been demonstrated in another toxic freshwater cyanobacterium, *Microcystis aeruginosa* (Harke *et al*. 2012; Harke & Gobler, 2013) and a range of marine cyanobacterial species (e.g. Lomas *et al*. 2014).

Recently, a study by Prentice *et al*. (in press) identified that the switch to active uptake could be predicted based on DIP concentrations in the water column (Fig. 1). In this study, a threshold of 4.7 µg L^{-1} DIP switched DIP uptake from passive to active. Temperature was a key factor controlling rates of active uptake.

It has also been demonstrated that *C.raciborskii* in DIP deficit is able to optimise it's growth based on low and short term phosphorus fluctuations (Wu *et al*. 2012; Amaral *et al.* 2014). Amaral *et al.* (2014) conclude that the frequency and duration of DIP additions was a more important factor driving *C. raciborskii* dominance than P concentration. Microcosm studies have also showed that daily DIP pulses can increase the dominance of *C. raciborskii* relative to other phytoplankton species (Posselt *et al.* 2009).

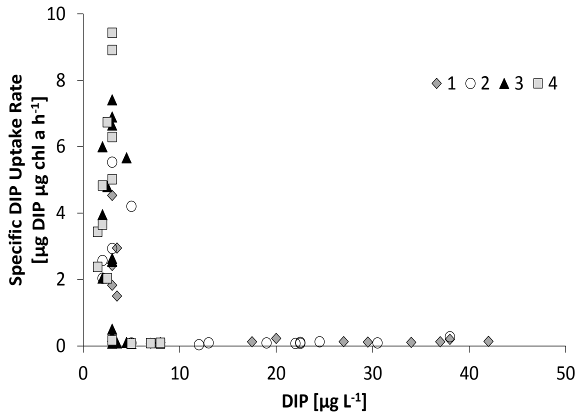

Fig. 1. Dissolved inorganic phosphorus (DIP) uptake (µgDIP µg $chla^{-1}$ h^{-1}) vs DIP concentrations (µg L^{-1}) at four sites in a subtropical reservoir over a one year period. Adapted from Prentice *et al.* (in press).

Like many other cyanobacterial species, *C. raciborskii* is able to utilize organic phosphorus sources to supplement DIP. Laboratory studies have shown the ability to utilize phosphorus esters, via measures of alkaline phosphatase activity (Posselt 2009; Fang Bai et al. 2014). However, more recently, genes for the use of other organic phosphorus sources, such as phosphonates, have also been identified (Stucken et al. 2010).

There also appears to be a relationship between phosphorus availability and concentrations of the *C. raciborskii* toxin, CYN. This relationship is not related to growth rate or phase since a number of studies have shown that CYN is constitutively produced (Davis et al. 2014; Stucken et al. 2014; Pierangelini et al. in press). A study in mesocosms with phytoplankton populations dominated by *C. raciborskii* has shed some light on the link between toxin concentrations and phosphorus additions (Burford et al. 2014). This study found that the addition of phosphorus resulted in a shift towards strains with higher CYN cell quotas (Fig. 2, Burford et al. 2014).

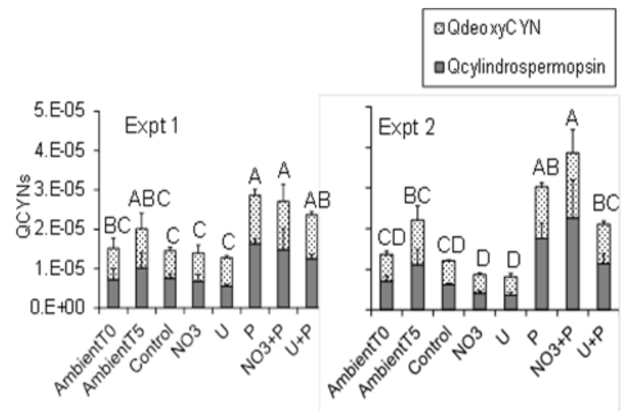

Fig. 2. CYN cell quotas (Q_{CYNs}) after five days of addition of nitrogen and/or phosphoris in mesocosom experiments. A,B,C,D shows significant differences (P<0.005). Adapted from Burford *et al.* (2014).

Laboratory studies have substantiated that *C. raciborskii* strains from the same waterbody can have a wide range of CYN cell quotas (A. Chuang, unpubl. Data). Indeed competition studies of strains of both *C. raciborskii* and *M. aeruginosa* in laboratory studies have shown that strains, rather than species dictate dominance under different phosphorus and light conditions (Marinho *et al.* 2013). The implication of this is that studies which focus on species-to-species differences have seriously oversimplified competition between cyanobacterial strains for resources.

Nitrogen

C. raciborskii has terminal heterocysts allowing it to fix nitrogen (Plominsky *et al.* 2013). There is considerable debate in the literature about the

importance of nitrogen fixation in driving cyanobacterial dominance in freshwater systems (Lewis & Wurtsbaugh, 2008; Schindler *et al.* 2008). However, Saker and Neilan (2001) showed that dissolved inorganic nitrogen sources resulted in higher growth rates than nitrogen fixation in *C. raciborskii* cultures. This is because dissolved nitrogen is a more energetically efficient nitrogen source.

A more recent study in a subtropical reservoir dominated by *C. raciborskii* showed dissolved inorganic nitrogen, rather than atmospheric nitrogen, was the dominant nitrogen supply most of the time (Burford *et al*. 2006). Additionally, urea can provide a source of nitrogen for growth (Burford *et al*. 2014). Moisander *et al*. (2012) had similar findings in their study of *C. raciborskii* in a subtropical river. They concluded that facultative diazotrophy gives *C. raciborskii* a competitive advantage under conditions of fluctuating nitrogen conditions. It has been proposed that activation of nitrogen fixation in *C. raciborskii* is dependent on the nitrogen content of the cells (Sprӧber *et al*. 2003).

Interesting, a study of the rise of *C. raciborskii* dominance in New Zealand showed that this coincided with higher nitrogen levels in the water column, rather than increased nitrogen fixation (Wood *et al.* 2014). Chislock *et al*. (2014) in their study of mesocosm study also found that *C. raciborskii* dominated irrespective of the nitrogen concentrations or nitrogen:phosphorus ratio. Therefore, overall it appears that a flexible response to nitrogen, rather than the use of nitrogen fixation alone, gives *C. raciborskii* a competitive advantage.

Modelling implications

The management of algal blooms in aquatic systems, including blooms of *C. raciborskii,* is aided by the use of algal growth models as a predictive tool. However, there is significant room for improvement in these models since predictive power remains low (Arhonditis & Brett 2004). There is also acknowledgement that despite their wide useage, state variables for models have changed little for decades (Glibert *et al.* 2010; Oliver *et al.* 2012; Robson, 2014). In part this is because these models: 1. have not incorporated new findings on algal physiology; 2. are not necessarily aligned with measured variables for algal biomass (Kara *et al.* 2012); and 3. do not take into account strain heterogeneity or the full range of nutrient forms, e.g. organic nutrient utilisation.

These new findings highlighted in this review identify the need for models that are validated with time-resolved changes in nutrient fluxes. The challenge is therefore to refine models such that they realistically represent diverse ecosystem components, while still incorporating individual harmful algal species (Harris *et al.* 1994).

This review has highlighted a number of strategies used by *C. raciborskii*. This includes:

- The ability to switch between active and passive DIP uptake. However models are typically based on classical hyperbolic uptake kinetics with increasing DIP concentrations (Dugdale 1967) which do not incorporate active uptake.

- The importance of fluctuating phosphorus conditions on growth. Once again the classical relationships between uptake and DIP concentrations do not capture this.

- The use of organic phosphorus as an alternative supply. The degree to which this is important in freshwater systems is unclear but models typically do not incorporate this, with few exceptions (Llebot et al. 2010).

- Alternating use of nitrogen fixation and dissolved nitrogen provides a competitive advantage under low and fluctuating nitrogen conditions.

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Can evolutionary ecology help us to design better biotoxin detection tools? The case of *Alexandrium*

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Abstract

An increase in the occurrence, frequency and severity of blooms of paralytic shellfish toxin (PST) producing *Alexandrium* species over the past 30 years has been reported from many parts of the world. PSTs impact disparate groups of marine organisms and can even structure ecosystems, leading to them being considered 'keystone metabolites'. There have been many hypotheses as to the role of PSTs for *Alexandrium* species: of these, a role as defensive compounds against copepod predation has been generally supported. This hypothesis would suggest that predation might have acted as a selective force in the evolution of toxicity in the genus. A study of the evolution and phylogeny of *Alexandrium* and the A1 and A4 domains of the *sxt*A gene , and *sxtG* gene can be used to investigate such selection. The information on genes related to saxitoxin synthesis in dinoflagellates can be applied to the design of rapid detection tools for marine biotoxin monitoring. Such tools have been shown to be useful in the quantification of blooms of *Alexandrium* species producing PSTs from Australian and New Zealand.

Keywords: Alexandrium, saxitoxins, *sxtA*, PSP, PST

Introduction

Bloom events of species of *Alexandrium* have increased in the recent past (Anderson *et al* 2012). New records have been made of *Alexandrium* species blooming from previously unaffected areas (Pitcher *et al* 2007, Farrell *et al* 2013, Burson *et al* 2014), and other areas in which blooms were formerly occasional, are now experiencing annual events (Fig. 1.).
The fact that some *Alexandrium* species produce paralytic shellfish toxins (PSTs) is the major reason for the unprecedented interest in the genus. Putative genes involved in the synthesis of PSTs in dinoflagellates have been discovered in the past few years (Stüken *et al*., 2011; Murray *et al.,* 2011a; Hii *et al*., 2012; Hackett *et al*., 2013; Orr *et al*., 2013; Suikkanen *et al*., 2013), following the first identification of these genes in cyanobacteria (Kellmann *et al*., 2008). A group of 14 'core' genes (*sxtA-sxtI, sxtP-sxtR, sxtS,* and *sxtU*) are conserved between the *sxt* clusters of cyanobacterial PST-producing strains (Murray *et al*., 2011b), of which eight (*sxtA, sxtB, sxtD, sxtG, sxtH or sxtT, sxtI, sxtS,* and *sxtU*) may be directly implicated in PST synthesis (Kellmann *et al*., 2008). All eight have now been identified from dinoflagellates (Stüken *et al*., 2011; Murray *et al*., 2011a; Hii *et al*., 2012; Hackett *et al*., 2013; Orr *et al*., 2013).

Almost all species of dinoflagellates that produce PSTs belong to the genus *Alexandrium*. Approximately 32 species have been described, and are present worldwide in comparable habitats (Balech, 1995; Anderson *et al*., 2012; Farrell *et al*., 2013; John *et al.,* 2014; Murray *et al*., 2014). In order to determine the evolution of PSTs within dinoflagellates, a well resolved phylogeny of the group, including the closely related PST producer *Pyrodinium bahamense*, is necessary. A recent phylogeny of the group with comprehensive species coverage, based on a concatenated alignment of three rRNA regions, found that clades within *Alexandrium* were generally not well supported (Murray *et al*., 2014). Many questions remain about the evolution of *Alexandrium*, as studies appear to be divided on which species is the most basal clade, and new cryptic species continue to be identified (John *et*

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al. 2003; Rogers *et al*., 2006, Leaw *et al*., 2007, Anderson *et al*. 2012, Orr *et al*., 2011), including *A. diversaporum* in 2014, (Murray *et al*., 2014) from an area of Australia in which previous studies had been conducted.

An analysis of the evolution of *Alexandrium* as compared to that of several of the key *sxt* genes can be used to evaluate the role of *sxt* genes in *Alexandrium* evolution. *Sxt* genes have also been targeted for molecular detection tools for blooms containing PSTs. Given that blooms of *Alexandrium* appear to be increasingly common, and are becoming established in many areas (Fig. 1), there is an increasing need for simple and rapid detection tools that can be used by shellfish farms and those without access to extensive scientific training or experience.

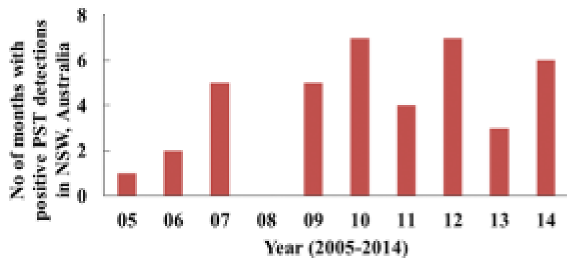

Fig. 1. Annual number of months in which closures of shellfish farms occurred due to PSTs, generally related to blooms of *Alexandrium pacificum*, in New South Wales, Australia, from 2005-2014 (Farrell *et al* in press). No closures were recorded in 2008.

Material and Methods

Cultures of *Alexandrium* were obtained from the following culture collections: the University of Tasmania culture collection, The National Centre for Culture of Marine Phytoplankton, Australian National Algae Culture Collection and the Cawthron Institute culture collection of microalgae. *Alexandrium* cells were harvested by centrifugation for 15 minutes at 4˚C at 3,220g (Eppendorf 5415R, Germany). DNA was extracted using a modified CTAB- phenol-chloroform method. Partial sequences of the rRNA genes LSU and SSU and complete 5.8s/ITS genes were amplified using previously published primers: SS3, SS5, D1R, D3B, and ITSfor, ITSrev (Murray *et al*., 2012). For the amplification of *sxtA* genes, *sxtA1* and *sxtA4* domains were amplified using the primers sxt001, sxt002 (A1) and sxt007 and sxt008 (A4) (Stüken *et al*., 2011). PCR reactions were conducted as described in Stüken *et al*., (2011) and Murray *et al* (2014).

Results and Discussion

Phylogenetic analyis of *Alexandrium* (Fig 2) showed that those species of *Alexandrium* which produce PSTs are distributed throughout the *Alexandrium* genus. These includes three of the five species that were formerly part of the *Alexandrium tamarense* species complex (John *et al* 2014a, 2014b), as well as *Alexandrium tamiyavanichi, A. minutum, A. ostenfeldii.*

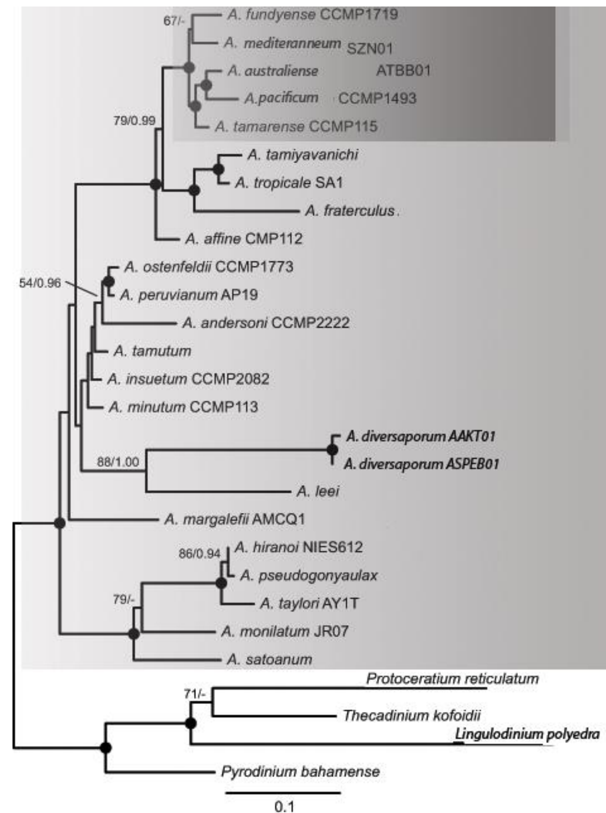

Fig. 2. Phylogeny of *Alexandrium* based on three concatenated regions of rRNA genes (3233 characters) inferred using Maximum Likelihood analysis (RaxML). The former *Alexandrium tamarense* species complex clade is highlighted, as are the species of the genus *Alexandrium*. Adapted from Murray *et al* 2014.

The analysis of the phylogeny of *sxtA1, sxtA4*, and *sxtG* (Fig 3) showed that they were each related to the homologous cyanobacterial genes. The *sxtA1* gene has an *Alexandrium* specific copy in addition to a copy that is also present with the STX producers *G. catenatum* and *P. bahamense*. A paralogous copy of sxtA1 is present in multiple dinoflagellate species, spanning all orders, including species within *Alexandrium*.

The *sxtA4* gene is only present in STX producing dinoflagellates. The gene is highly conserved between species, resulting in the lack of a branching pattern between species.
The *sxtG* gene is present in both stx producing and non-producing *Alexandrium* strains in addition to *G. catenatum* and *P. bahamense*. There is a distant relationship to cyanobacterial cyrA, with the possible presence of a dinoflagellate cyrA paralog in multiple species, spanning numerous orders.

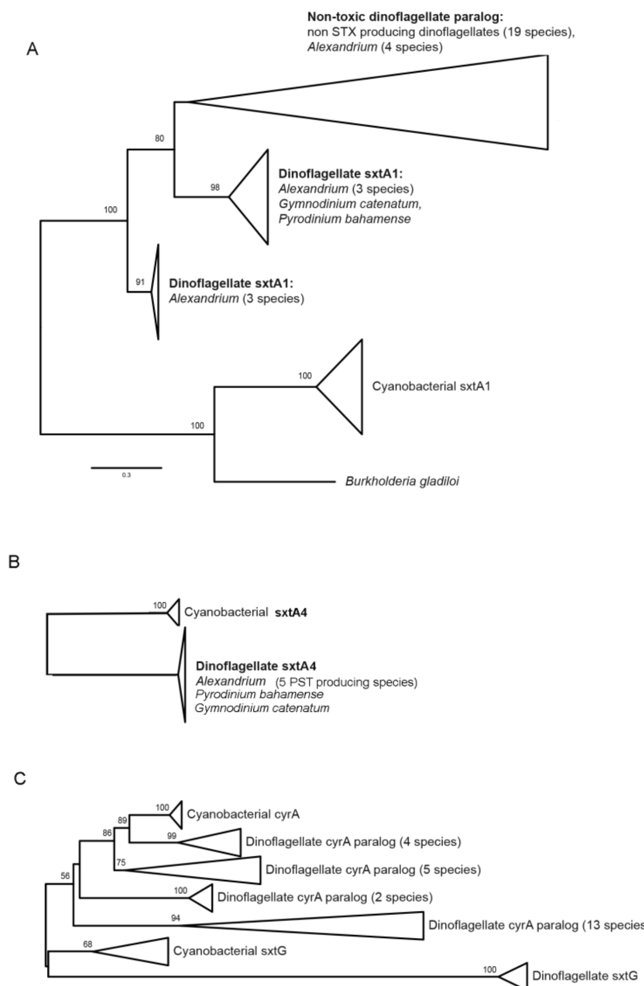

Fig. 3. A Phylogeny of *sxtA1*. B Phylogeny of *sxtA4*. C. Phylogeny of *sxtG*.

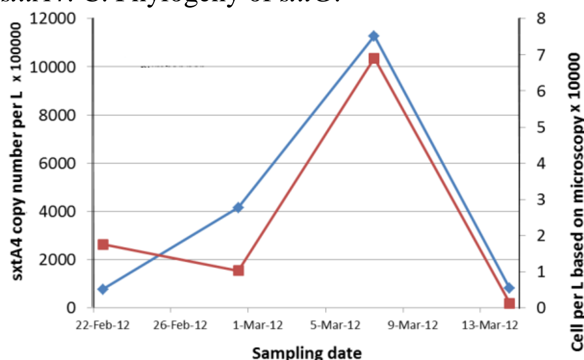

Fig. 4. Abundance of *sxtA4* gene copies (primary y axis) and estimates of *Alexandrium pacificum* (secondary y axis) based on cell identifications and counts using a microscope, from Marlborough Sounds, New Zealand, during March 2012.

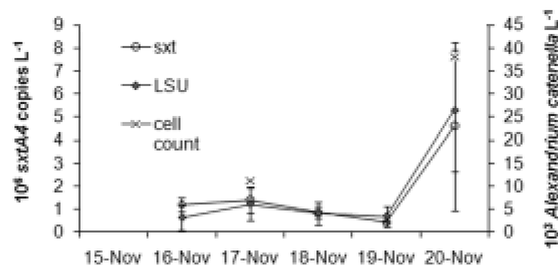

Fig. 5. Abundance of *sxtA4* gene copies (primary y axis) and estimates of *Alexandrium pacificum* (secondary y axis) based on cell identifications and counts using a microscope, as well as cell enumeration using LSU rDNA qPCR, at Botany Bay, Sydney Australia, during November 2010 (Murray *et al*. 2011a).

The analysis of phytoplankton samples directly, using a qPCR primer pair targeted at *sxtA4* (Murray *et al* 2011) was conducted on phytoplankton samples from Australian and New Zealand sites (Figs 4, 5). during known blooms of *Alexandrium pacificum* (Litaker). These analyses showed that this method was generally closely correlated with the cell abundance of *Alexandrium pacificum* as estimated from counts using a light microscope (Figs 4, 5). The *sxtA4* gene was not amplified from non-PST-producing *Alexandrium* species but was amplified from the relatively distantly related PST-producing species *Gymnodinium catenatum* (Murray *et al* 2011).
This shows potential as a single assay to detect distantly related potential PST-producing taxa, including those not previously known from a particular site. The expansion of this method for the detection of PST-producing taxa within shellfish tissue, as a rapid screen for possible PST positives, is clear, and requires more in depth investigation.

Acknowledgements

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Preparation of Diarrhetic Shellfish Toxins (DSTs) and Paralytic Shellfish Toxins (PSTs) by Large Algal Culture and Chemical Conversion

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Abstract

Okadaic acid (OA) and dinophysistoxin-1 (DTX1) were prepared from *Prorocentrum lima*. Suitable strains were selected and the culture conditions were optimized. To prepare paralytic shellfish toxin (PST) calibration standards, C1/2 toxins produced by the cyanobacteria *Anabaena circinalis* were used to chemically produce gonyautoxin-2/3 (GTX2/3) (using heat and 0.1M HCl), GTX5 (reduction using 1% DTT), and decarbamoylgonyautoxin-2/3 (dcGTX2/3) (using heat at pH 7.0). GTX5 was further converted to decarbamoylsaxitoxin (dcSTX) by hydrolysis. GTX1/4 were produced by large cultures of *Alexandrium tamarense*. The reductive elimination of 11-O-sulfate group in GTX1/4 by DTT yielded neosaxitoxin (neoSTX). All reaction conditions were optimized, to enable the production of the major PSTs starting from cultured cells.

Keywords: Anabaena circinalis, Okadaic acid, Diarrhetic shellfish toxins, Dinophysistoxin-1, Paralytic shellfish toxins, *Prorocentrum lima*, Saxitoxin analogues

Introduction

Marine toxins in shellfish have been tested by the mouse bioassay (MBA) in many countries including Japan. Recently several alternative testing methods have been developed. The most widely accepted method is liquid chromatography (LC) with mass spectrometry (MS) or fluorescent detection (FD) because these methods are superior to the MBA in sensitivity and accuracy (Quilliam 2003; Szuuki and Quilliam 2011). Accurate calibration standards are essential to validate instrumental methods. Several certified reference materials are available from the National Research Council of Canada (NRC) Biotoxin Metrology Program. This program has contributed to a worldwide routine monitoring of marine toxins. On the other hand, several calibration standards of shellfish toxins including diarrhetic shellfish toxins (DSTs), okadaic acid (OA) and dinophysistoxin-1 (DTX1), other lipophilic toxins pectenotoxin-1,2,6 (PTX1,2,6) and yessotoxin (YTX), paralytic shellfish toxins (PSTs) have been prepared in several research programs in Japan (Suzuki and Watanabe, 2011).

With the change in the definition of DSTs in Japan in April 2015, the MBA as the Japanese official testing method for DSTs was replaced by instrumental methods including LC/MS/MS to detect OA analogues exclusively. This will result a huge demand for DST calibration standards in Japan. In this review, we describe the preparation of OA and DTX1 by large algal cultures. Another important toxin group within the Japanese shellfish toxin monitoring program are the PSTs. Although the MBA is still the official testing method for PSTs in Japan at present, we have also been investigating efficient preparation methods of a range of purified PSTs. In the present review, the preparation of PSTs by large algal cultures followed by chemical conversions is also described.

Preparation of DSTs (OA and DTX1)

OA and DTX1 were prepared from *Prorocentrum lima* isolated from Okinawa islands, Japan. Of 85

A. Lincoln MacKenzie [Ed]. *Marine and Freshwater Harmful Algae 2014*. Proceedings of the 16th International Conference on Harmful. Algae. Cawthron Institute, Nelson, New Zealand and the International Society for the Study of Harmful Algae (ISSHA)

strains isolated around the Okinawa islands, 67 subcultures producing OA were maintained in our culture collections. Of these 67 strains, DTX1 was detected in 50 strains. The cellular toxin content of OA and DTX1 in the 50 initial subcultures ranged from 0.01-0.42 and 0.01-0.21 pg/cell, respectively. Although these values were lower than those reported for *P. lima* previously collected in Okinawa, Japan (Lee *et al.* 1989), a strain (PL197) with a suitable growth rate and high toxin contents of OA and DTX1 was chosen for a large algal culture source.

Fig. 1 shows the large scale culture system for *P. lima*. *P. lima* was cultured in a series of 20 L boxes with ES-1 medium at 25 °C under a plant cultivation red light (14 hours light/10 hours dark cycle). The culture medium was circulated through the system and toxins excreted from cells were collected by passing the medium through the HP 20 resin. In our initial survey to optimize the culture conditions, a strain of *P. lima* (PL197) producing both OA and DTX1 was cultured at two different temperatures. The highest cell densities were obtained for cultivation periods between 80 and 100 days at 25 °C (Fig. 2). Although the DTX1 cellular content in the *P. lima* strain (PL197) was lower than that obtained for OA, the cellular content of DTX1 increased with the cultivation period as shown in Fig. 3. The cellular contents of DTX1 and OA obtained at 25 °C were higher than those obtained at 20 °C, therefore the *P. lima* strain (PL197) was cultured at 25 °C.

Fig. 1. A large culture system of *P. lima.*

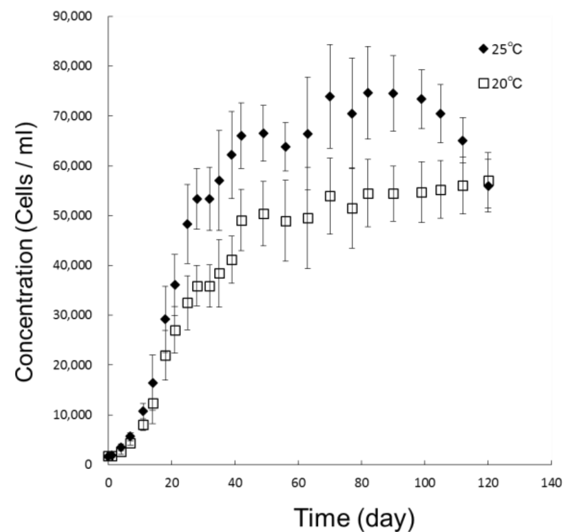

Fig. 2. Culture growth (n=4) of *P. lima* strain (PL197) at different temperatures.

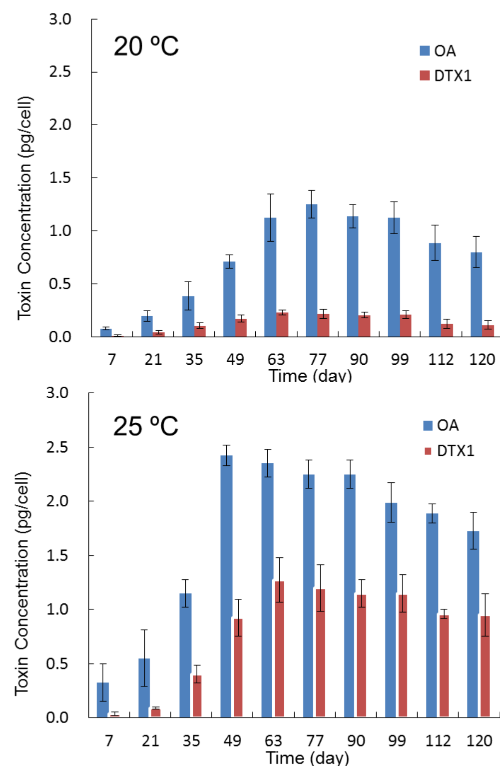

Fig. 3. Changes in the cellular toxin content (pg/cell) (n=4) of *P. lima* (PL197) with the length of incubation.

Fig. 4 shows the isolation scheme of OA and DTX1 from large cultures of *P. lima.* The isolation scheme basically followed that reported previously by Goto *et al.* (1998) with some modifications. OA and DTX1 absorbed on the HP 20 resin from the culture medium were

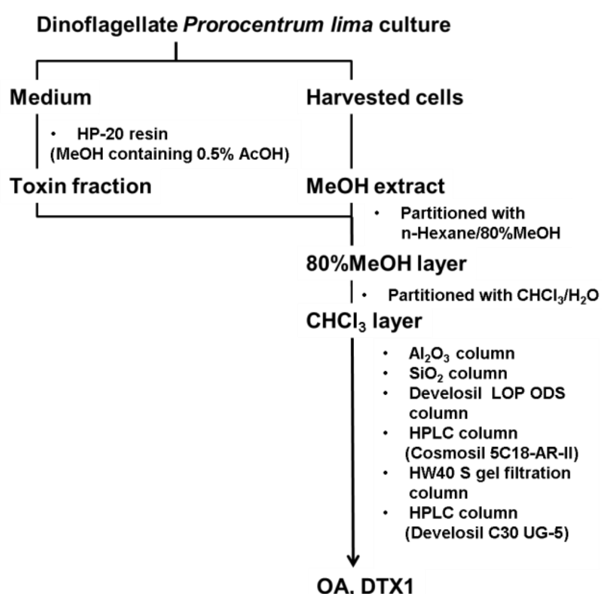

Fig. 4. Isolation scheme of OA and DTX1 from *P. lima* culture extracts.

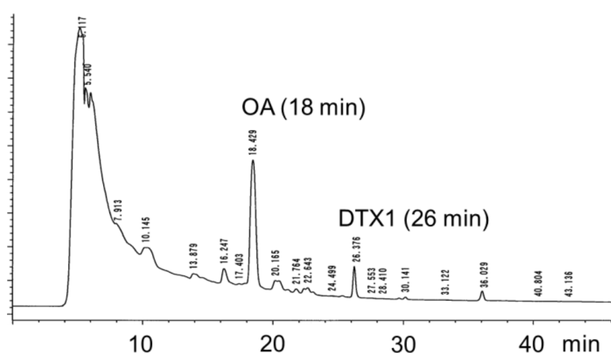

Fig. 5. HPLC/UV chromatogram of OA and DTX1 obtained on the Cosmosil 5C18-AR-II (10 mm i.d. x 250 mm).

extracted by washing the resin with methanol containing 0.5% acetic acid and this was combined with methanol extracts of harvested cells. Toxin extracts were partitioned between n-hexane and aqueous methanol followed by a partition between chloroform and water. The extract partitioned into the chloroform layer was evaporated and chromatographed on an open glass alumina oxide column with dichloromethane-methanol (1:1, v/v), followed by methanol, 1% ammonium/distilled water (v/v)-methanol (1:1, v/v). The toxin fraction eluted with 1% ammonium/distilled water (v/v)-methanol (1:1, v/v) was evaporated and chromatographed on an open glass silica gel column using dichloromethane-methanol (95:5, v/v), (1:1, v/v), and methanol. The toxin fraction eluted with dichloromethane-methanol (1:1, v/v) was evaporated and chromatographed on Develosil LOP ODS (Nomura Chemical, Japan) medium pressure column (20 mm i.d. x 200 mm) using an increasing aqueous methanol to methanol stepwise gradient. OA and DTX1 eluted with methanol-distilled water (85:15, v/v) were finally isolated by HPLC on Cosmosil 5C18-AR-II (10 mm i.d. x 250 mm, Nacalai Tesque, Japan). HPLC eluent A was Milli-Q water and B was acetonitrile, both containing 0.1% acetic acid. Gradient elution from 55% to 100% B was performed over 45 min at a flow rate of 2.3 mL/min. OA and DTX1 were monitored by UV at 200 nm.

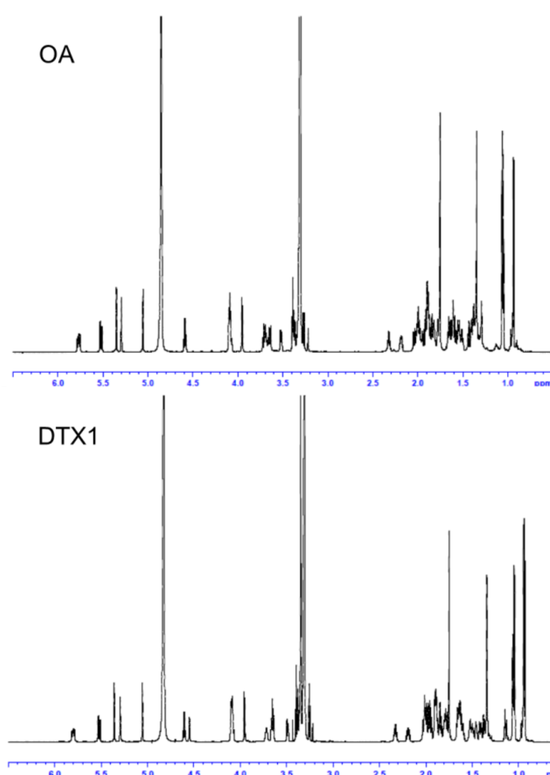

Fig. 6. ^{1}H-NMR of OA and DTX1 isolated from *P. lima*.

Fig. 5 shows the HPLC/UV chromatogram of OA and DTX1 obtained on the Cosmosil 5C18-AR-II preparative column. If the purity of OA and DTX1 was insufficient, they were re-chromatographed on HW 40 S column (10 mm i.d. x 300 mm, Tosoh, Japan) with methanol at a flow rate of 1 mL/min. Toxins were finally isolated by HPLC on the Develosil C30 UG-5 (4.6 mm i.d. x 250 mm, Nomura Chemical, Japan) with acetonitrile-distilled water (7:3, v/v) containing 0.5% acetic acid at a flow rate of 1 mL/min. Fig. 6 shows the ^{1}H-NMR spectrum of OA and DTX1, respectively. Two hundred vials of certified reference material (CRM) of OA and

DTX1 containing 0.5 mL at 1 ppm solution were prepared and distributed to 84 laboratories in Japan in February 2015.

Table 1. CRM-OA and -DTX1 provided to 84 laboratories in Japan.

	CAS No	Conc. (mg/L)	Solvent	Expanded Measurement Uncertainty (k=2)(mg/L)
OA	78111-17-8	0.99	0.5% EtOH/MeOH	0.06
DTX1	81720-10-7	1.09	0.5% EtOH/MeOH	0.09

Table 1 lists the specifications of the CRM-OA and CRM-DTX1 prepared in 2015. The final OA and DTX1 CRMs will be commercially available from 2016. As part of our research program we have also developed a chemically and microbiologically stable scallop homogenate CRM, naturally contaminated with DTX1 and DTX3. Due to the absence of OA in the natural homogenate, the CRM was fortified with OA.

Preparation of PSTs (C1/2, GTX1/2/3/4, dcGTX2/3, dcSTX, neoSTX)

A calibration standard of C1/2 was prepared from large algal cultures of *Anabaena circinalis* (CS-541/04) following a method previously described by Watanabe *et al.* (2011). Fig. 7 shows the large scale *A. circinalis* culture system. *A. circinalis* was cultured in a series of 10 L plastic bottles with Fitzgerald medium at 17 °C. Cultures were incubated at 17°C under a 16 hour light/8 hour dark cycle with illumination at 8-15 μmol/m^2/s.

Fig. 8 shows the isolation scheme of C1/2 from the *A. circinalis* culture. C1/2 were extracted with 0.5 M acetic acid and the extract was passed through an open glass column packed with activated charcoal (Wako Chemicals, Japan). The column was washed with 0.05 M acetic acid then C1/2 were eluted with 4% acetic acid/distilled water-ethanol (1:1, v/v). In the isolation procedure reported in a previous study (Goto *et al.* 1998), the toxin fraction was chromatographed on the Bio Gel P2 column. We found it difficult to get reproducible results to improve the purity of C1/2 using this method, therefore purification of C1/2 on a membrane filter unit (Vivaflow 50) composed of regenerated cellulose (10000 MW cut-off, Sartorius, Germany) was investigated.

Fig. 7. A large culture system of *A. circinalis.*

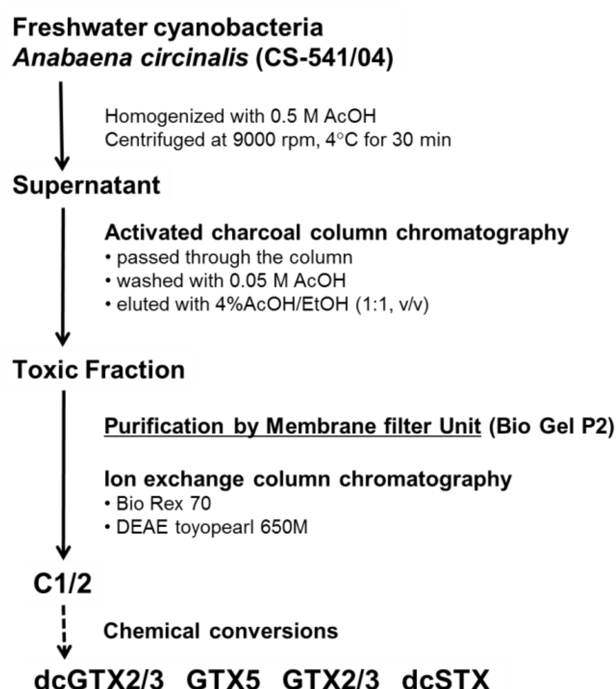

Fig. 8. Isolation scheme of C1/2 from *A. circinalis* culture extracts.

Fig. 9 shows the process of purification of C1/2 using the membrane filter unit. Extracts eluted from the activated charcoal column with 4% acetic acid/distilled water-ethanol (1:1, v/v) were applied to the membrane filter unit. By using the membrane filter unit, thick and dark green colored extracts were purified to transparent extracts (Fig. 9). Eight hundred mL of toxin extracts eluted from the activated charcoal column corresponding to 30 L culture extracts can be filtered. It took about 1 day to complete the filtration and this was far shorter and easier than the conventional gel filtration chromatographic procedure on the Bio Gel P2. Fig. 10 shows the recovery of C1/2

obtained after purification with the membrane filter unit. Fairly good recovery of C1/2 of more than 90 % was obtained. After the filtration of the extracts, the membrane filter was washed with 0.05 M acetic acid twice. C1/2 recovered from the membrane units by duplicate washes was approximately 4 %.

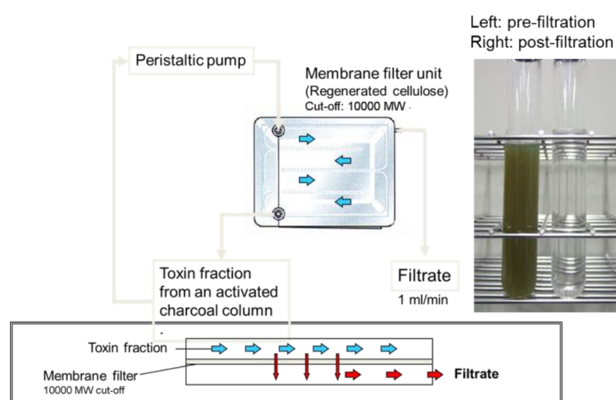

Fig. 9. Diagram of purification of extracts using a membrane filter unit (Vivaflow 50).

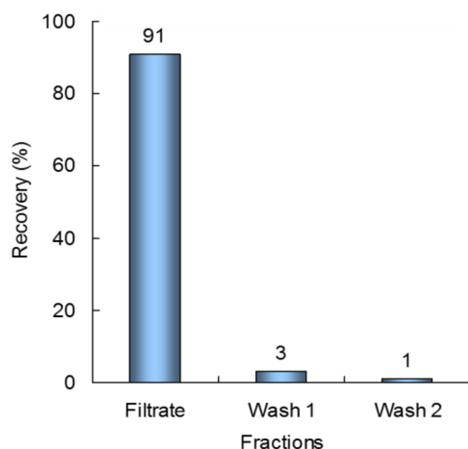

Fig. 10. Recovery of C1/2 from the extracts using a membrane filter unit.

The filtrate containing C1/2 was chromatographed on the Bio Rex 70 (Bio-Rad, USA) column followed by DEAE Toyopearl 650 M (Tosoh, Japan) column by using 0.05 M acetic acid as a mobile phase. Fig. 11 shows the ^{1}H-NMR spectrum of C1/2 isolated from *A. circinalis*. The purity of C1/2 obtained by our present isolation procedure was comparable or better than that obtained by a previous method (Goto *et al.* 1998).

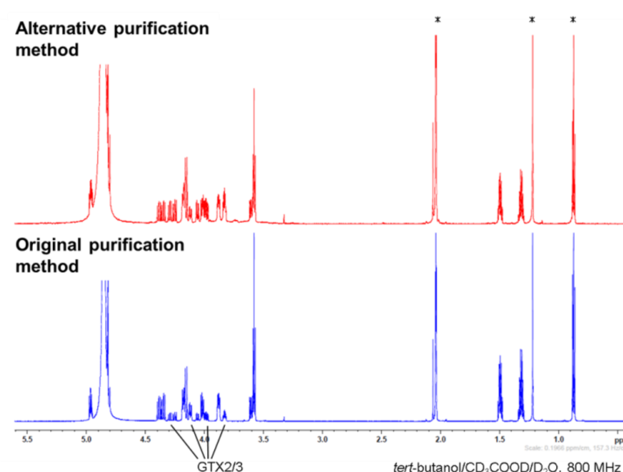

Fig. 11. ^{1}H-NMR spectrum of C1/2 isolated from *A. circinalis*.

In our previous study (Watanabe *et al.* 2011), C1/2 were chemically converted to dcGTX2/3, GTX5, GTX2/3 and dcSTX. Although the chemical conversions of C1/2 were simple and rapid, low recoveries of dcGTX2/3 and GTX5 were problematic. To improve these poor recoveries of toxins in the chemical conversions of C1/2, the reaction conditions were re-investigated. Fig. 12 shows preparation scheme of several PSTs. Reaction conditions provided by red and blue letters are our newly developed conditions. Approximately 100% of C1/2 was converted to GTX5 in our present method. More detailed results will be reported elsewhere.

Due to difficulties with the chemical conversion of C1/2 to GTX1/4, GTX1/4 were prepared from large algal cultures of *Alexandrium tamarense*. Fig. 13 shows the PST profiles obtained from several strains of *A. tamarense* collected from Ofunato Bay, Japan. The percentage proportions of PSTs differed among strains. Similar variations in toxin profiles of *A. tamarense* collected from Tohoku coastal area in Japan were observed in a previous study (Ichimi *et al.* 2002). A strain (OF-23) producing a high proportion of GTX1/4 and absence of C1/2 in the toxin profile was suitable to isolate GTX1/4. neoSTX was chemically converted from GTX1/4 with a high recovery (Fig. 12).

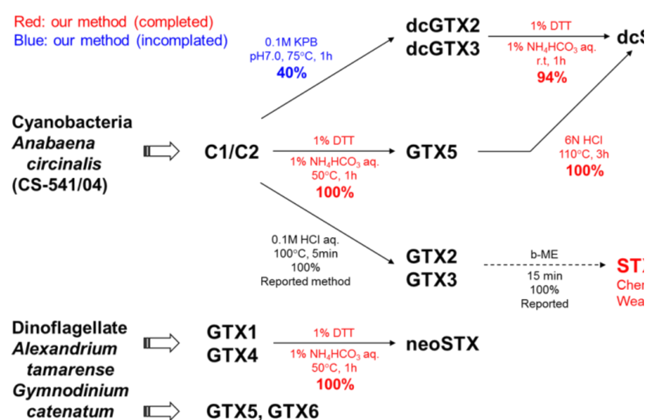

Fig. 12. Preparation scheme of saxitoxin analogues by chemical conversion.

To isolate GTX5 and GTX6, several strains of *Gymnodinium catenatum* were collected from Inokushi Bay, Japan. Some strains produced GTX5 and GTX6 as the dominant toxins and the sum of them exceeded more than 60% of the total toxins. This made them suitable for the isolation of GTX5 and GTX6. More detailed results on the toxin profiles of *A. tamarese* and *G. catenatum* collected in our research program will be reported elsewhere.

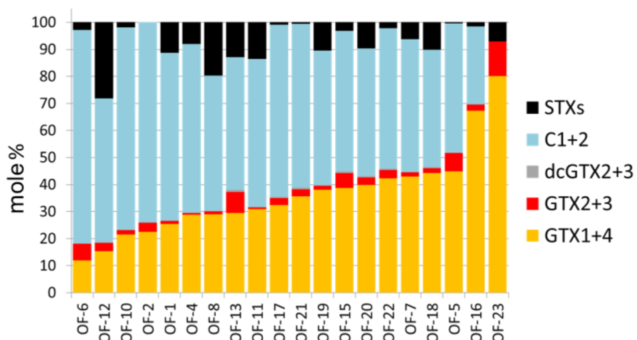

Fig. 13. PST profiles obtained from several strains of *A. tamarense* collected from Ofunato Bay Japan.

Conclusions

With the change in the definition of DSTs in Japan, the MBA as the Japanese official testing method for DSTs was replaced by instrumental methods, including LC/MS/MS, to detect OA analogues exclusively. Calibration standards of OA and DTX1 were prepared from large algal cultures of *P. lima* by the national research program in Japan and CRM-OA and -DTX1 were provided to 84 laboratories involved in DST monitoring program. Although our CRM will be provided to domestic laboratories in Japan, they might also contribute to worldwide shellfish toxin monitoring programs in a case of shortage of commercial CRM. Almost all of the PST calibration standards were prepared by large cultures and chemical conversions. Development of CRM-PSTs has been necessary due to the planned adoption of instrumental testing methods for PSTs in Japan in the future.

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Risk assessment of seafood toxins

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Abstract

Marine microalgae are an important food source for shellfish. Many such algae produce complex secondary metabolites, some of which are highly toxic to mammals, and after ingestion these may be absorbed and stored in the tissues of the shellfish. These may then poison animals higher up the food chain, and many cases of shellfish poisoning in shellfish consumers are reported each year. In order to minimise the likelihood of such events, assessment of the risk of seafood toxins is required so that regulatory limits for the toxins can be put in place. While good progress has been made in the risk assessment of seafood toxins, more work is needed in some areas in order to provide the necessary data for setting appropriate regulatory limits.

Keywords: Algal toxins ; Seafood poisoning; Toxicology; Risk assessment

Introduction

Many secondary metabolites are synthesised by marine microalgae, some of which are highly toxic to mammals. Such algae are a major food source of shellfish and the toxins that they produce may become concentrated in the tissues of the seafood, leading to toxic effects in consumers. Uptake by shellfish cannot be avoided, and there is presently no fully-effective method for removing toxins from shellfish after harvesting. From time to time, therefore, contamination of seafood with toxins is inevitable, and in order to avoid adverse effects on human health, we must ensure that the levels of such contaminants are below those known to cause toxicity. Such levels are determined by the process of risk assessment, which permits the establishment of regulatory limits for seafood toxins, and seafood producers must ensure that the concentration of contaminants are not above such limits in product offered for human consumption.

Risk and risk assessment.

Risk may be defined as "the probability that an adverse effect will be induced in an individual through contact with a particular substance". Risk is a function of the amount of the chemical ingested and its intrinsic toxicity. A small amount of a highly toxic chemical will cause harm, as will a large amount of a chemical of relatively low toxicity. For risk assessment, it is therefore necessary to determine the levels of a particular toxin in shellfish and its intrinsic toxicity. It is a basic principle of toxicology that for any poison there is a dose below which it will have no perceptible adverse effects on the organism. This is defined as the No Observable Adverse Effect Level (NOAEL).

Risk assessment can be divided into two major areas, concerning the effects seen after acute exposure to a toxin, and those induced by prolonged ingestion of a toxic substance.

The parameter required for setting regulatory limits for acute exposure to a harmful substance is the Acute Reference Dose (ARfD), defined as the amount of a chemical in food that, in the light of present knowledge, can be consumed in the course of a day or at a single meal with no adverse effects. The parameter for chronic exposure to a harmful substance is the Tolerable Daily Intake (TDI), defined as the amount of a chemical in food that, in the light of present knowledge, can be consumed daily over a lifetime with no adverse effects (Benford, 2000; FAO, 2002).

Acute effects of seafood contaminants.

Ideally, the data for establishing an ARfD for a seafood contaminant would be based on human experience in outbreaks of poisoning, in which some consumers may show toxic effects and others none. For this, it would be necessary to obtain samples of the food eaten by individuals who ate the contaminated product, to have an accurate estimate of the amount of seafood eaten by each individual and a good analytical method for the toxin or toxins involved. Such criteria have

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rarely been achieved. For example, data obtained after outbreaks of paralytic shellfish poisoning have given overlapping estimates of the amounts of toxin causing no effect, moderate toxicity or death (EFSA, 2009). Now that modern analytical methods for shellfish toxins are available, further work in this area would be very valuable for risk assessment.

In the absence of good data on humans, we have to rely on animal experimentation for determining ARfDs. This involves determination of acute toxicities, most commonly with mice as the experimental animal. For this, we see the classic S-shaped curve (Figure 1), from which the LD_{100}, the LD_{50}, the LD_0 and the NOAEL can be obtained.

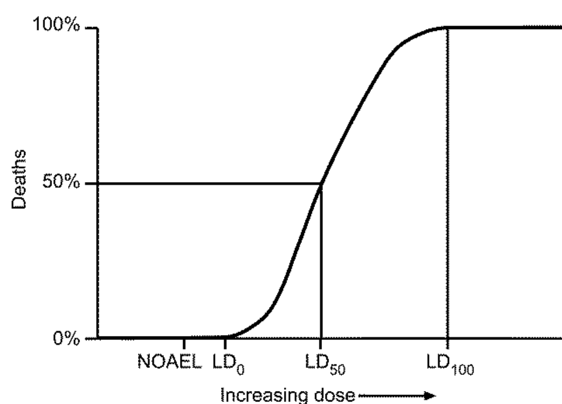

Fig. 1. Acute toxicity – dose-response curve

By application of a safety factor, the ARfD can be obtained from the NOAEL. The default factor is 100, which is based on the assumption that humans are 10 times more susceptible to poisons than mice and that the variability in response among humans is 10 times greater than that among these animals. Higher or lower safety factors may be used by regulatory authorities, however, depending upon the nature and quality of the data available for risk assessment.

It would seem obvious that toxicity studies in animals should employ the same route of exposure as that through which humans are exposed to the toxin, which, in most cases, is the oral route. This has not, however, always been the case, and some risk assessments have been based on toxicity by intraperitoneal injection. This would not be a problem if toxicity by intraperitoneal injection correlated with that by oral administration. But, as shown in Table 1, this is by no means the case. Ratios range from more than 700 for palytoxin to only 2 for pinnatoxin F.

Table 1. Lack of correlation between acute toxicity by i.p. injection and that by gavage.

Compound	Ratio of LD_{50} by gavage/LD_{50} by i.p. injection	Reference
Palytoxin	708	Munday, 2006
Gymnodimine	79	Munday *et al.*, 2004
13-Desmethyl spirolide C	23	Munday *et al.*, 2012a
Pinnatoxin F	2	Munday *et al.*, 2102b

The most widely-used technique for oral administration of materials to mice is gavage, in which a narrow tube is inserted into the oesophagus and a solution of the test substance released to flow into the stomach. There is evidence, however, that because of the semi-solid contents of the mouse stomach, the solution may flow around this material and rapidly enter the duodenum, the major site for absorption of toxins (Munday, 2014). This is different from the situation in humans, in which the liquid nature of the stomach contents ensures that toxins are mixed with the rest of the contents, from which they are slowly released into the duodenum. The human situation may be replicated in mice by administration of the toxin in a small amount of food palatable to the animal. This is eaten within a few seconds, and the toxin is mixed with the stomach contents, as in the human situation. As would be expected, LD_{50} values by feeding are higher than those by gavage (Table 2). Feeding may well be the most appropriate method of administration of toxins for risk assessment

Table 2. Comparative toxicity of algal toxins by gavage and by feeding.

Compound	Ratio of LD_{50} by feeding/LD_{50} by gavage	Reference
Saxitoxin	5.9	Munday *et al.,* 2013
Neosaxitoxin	4.6	Munday *et al.,* 2013
Spirolide C	4.3	Munday *et al.,* 2012a
Pinnatoxin F	2.0	Munday *et al.,* 2102b
Pinnatoxin G	2.4	Munday *et al.,* 2102b

Chronic effects of seafood contaminants.

Comparatively little information on the chronic toxicity of seafood toxins is available. For estimation of a TDI, short-term, sub-chronic and chronic feeding studies, along with investigation of possible effects on reproduction, are required. It is possible that regulatory authorities may in the future require chronic toxicity studies on seafood contaminants, but because the required tests are long-term and extremely expensive, it will be essential to prioritise such studies. One approach may be to give precedence to compounds that have been shown to be comparatively slowly eliminated from animals and humans after acute exposure, since in this situation repeat dosing may lead to build-up of the toxin or its metabolites, with consequent toxic effects.

Mechanism of toxicity of seafood toxins

An understanding of the mechanism of toxicity of seafood toxins is of considerable value in identifying individuals who may be at particularly high risk of intoxication, and in identifying effective therapies after intoxication. Studies on *in vitro* biochemical changes induced by seafood toxins have been conducted, and in many cases it has been assumed, without any corroborating evidence, that the changes seen *in vitro* are responsible for the toxic effects *in vivo*. This is a *post hoc ergo propter hoc* logical fallacy. For proper evaluation of mechanisms of toxicity, it is necessary to demonstrate that *in vitro* changes are also seen *in vivo* or *ex vivo* and that there is a defined pathway from the biochemical change *in vitro* to toxicity *in vivo*. Furthermore, it should be shown that other compounds that cause the same biochemical change *in vitro* induce the same toxic effects in animals.

In some cases, particularly those relating to the effect of toxins on neuromuscular transmission, these criteria have been met. Certain shellfish toxins and many other natural products of diverse chemical structure produced by bacteria, plants, spiders, reptiles and amphibians, inhibit neuromuscular transmission, leading to death by asphyxia. Such convergent evolution in the way in which organisms target predators and competitors is remarkable (Munday, 2014).

Conclusion.

While considerable progress has been made in the risk assessment of seafood toxins, there is still work to be done, particularly with regard to route of administration. Of particular concern is the situation with the paralytic shellfish poisons (PSPs), of which more than 50 congeners are known. Precise chemical methods for estimating the levels of many of these congeners in seafood are now available, but for risk assessment it will be necessary to have relevant and accurate data on the relative toxicity of the different compounds, so that an estimate of the overall toxic potential can be made. At present, data on the relative toxicities of PSPs are derived from a mouse bioassay involving intraperitoneal injection. Recent studies have shown that these data do not correlate with the acute oral toxicities of PSPs, requiring re-evaluation of the regulatory limits set for these toxins (Munday *et al.,* 2012a).

The primary objective of work in this area should be risk assessment based on sound science so that appropriate regulatory limits can be set. Such limits must neither be set too high, so that human health is endangered, nor set too low so that they unnecessarily disadvantage the seafood industry.

Acknowledgements

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Biogeography and toxicity of *Gambierdiscus* species

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Abstract

The dynamic state of biogeographical, physiological and toxicological research on *Gambierdiscus* has been greatly assisted by the availability of DNA sequence information and PCR assays. These allow rapid identification of taxa, facilitate comparisons of species suites from different locations and habitats and extend the known range of many species. With questions of identification, distribution and abundance largely resolved, sorting out the toxicity among *Gambierdiscus* species (isolates) is the next, crucial data layer in a functional biogeography of the genus.

Keywords: Ciguatoxin, haemolytic assay, maitotoxin, receptor binding assay, liquid chromatography–mass spectrometry

Introduction

The study of toxic cells from the genus, which would be described by Adachi and Fukuyo in 1979 as *Gambierdiscus,* was initially driven by chemists who sought the source of the toxins responsible for causing ciguatera fish poisoning (CFP). In 1967, Scheuer *et al.* isolated and characterized the first "ciguatoxin" from moray eels (*Gymnothorax javanicus*) which they credited with causing CFP. Over the ensuing decades, the methods for isolating and characterizing ciguatoxins (CTX) became more sophisticated, but the actual source of toxins remained unknown. Randall (1959) and later Halstead (1967) hypothesized that toxins were introduced into the marine food web when herbivorous fish consumed toxic microalgae and in turn were consumed by larger omnivorous or predatory fish. However, it was a full decade after the initial chemical characterization before Yasumoto and his colleagues found a dinoflagellate they believed was "the likely culprit of ciguatera" (Yasumoto *et al.* 1977). Shortly afterward, the anterio-posteriorly compressed dinoflagellate was named *Gambierdiscus toxicus* (Fukuyo and Adachi), in honor of the Gambier Islands French Polynesia, the type location.

The genus remained monotypic (*G. toxicus*) until Faust discovered *G. belizeanus* from the western Caribbean in 1995. In 1998 Holmes added *G. yasumotoi* from Pulau Hantu (Singapore). The globular or rounded form of *G. yasumotoi* caused some to question if it belonged in the genus *Gambierdiscus* or should be assigned to a new genus (Litaker et al. 2009). By1999 the brilliant pairing of Chinain and Faust (molecular biologist and morphologist) provided descriptions of *G. australes* (Australes Archipelago, French Polynesia [=FP]), *G. pacificus* (Tuamotu Archipelago, FP) and *G. polynesiensis* (Australes and Tuamotu Archipelagos, FP). Their SEMs and morphological descriptions were substantiated by sequence data. In 2009, Litaker working with Faust, Chinain and others revised the genus adding three new species from Belize, *G. caribaeus, G. carpenteri* and *G. ruetzleri* as well as *G. carolinianus* from the coast of North Carolina. This study provided light micrographs and SEMs for all described species and added the first *Gambierdiscus* sequence data to GenBank. It should be noted that *G. ruetzleri*'s morphology was similar to the globular *G. yasumotoi* rather than the anterio-posteriorly compressed shape of the other members of this genus. The emergent species suite was joined by *G. excentricus* and *G. silvae* described by Fraga and colleagues in 2011 and 2014 from the Canary Islands (NE Atlantic). *Gambierdiscus scabrosus* from Japan is the most recently described species in the genus (Nishimura *et al*. 2014). Both Tenerife, one of the Canary Islands in the northeast Atlantic (28°34'N), the type location for *G. excentricus*, and Honshui Island Japan, the type location for *G. scabrosus* (35°21'N), are north of areas typically considered habitat for *Gambierdiscus* until recent discoveries (Fraga *et al*. 2011, Nishimura *et al*. 2014). With the list of new *Gambierdiscus* species expanding rapidly, it seems likely this genus may

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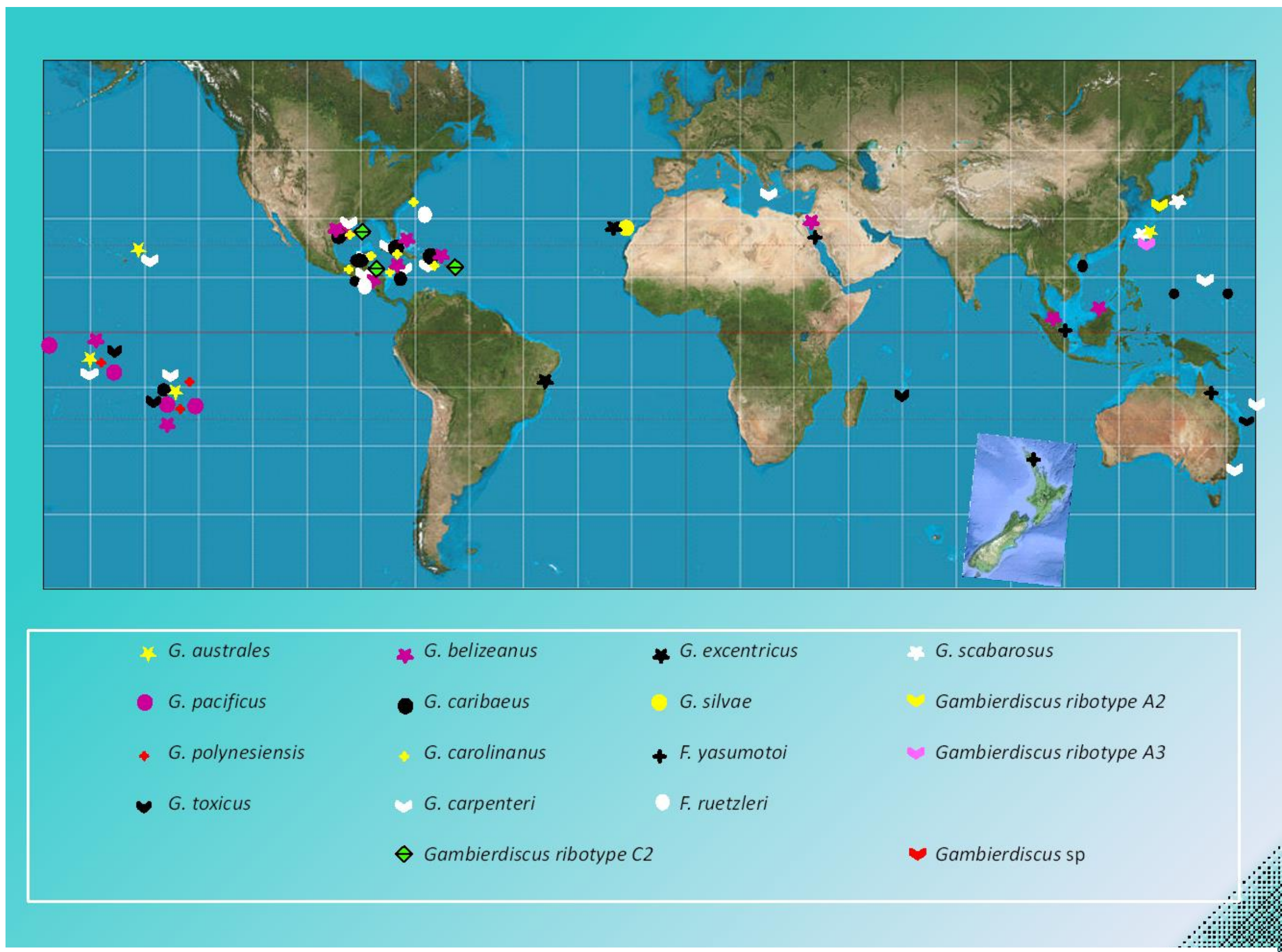

Fig. 1. *Gambierdiscus* and *Fukuyoa* species distribution. Note New Zealand is an inset. It was not represented in the original projection of this map.

no longer be characterized as tropical / subtropical. It should be noted that in an active area of research, it is not unexpected that taxonomic revisions will be necessary. In 2015 Gomez *et al*. proposed a new genus, *Fukuyoa* for the two globular *Gambierdiscus* species, *G. yasumotoi* and *G. ruetzleri*. This new taxonomy is reflected in Table 1 and the species distribution map (Fig. 1). Since the taxonomy of *Gambierdiscus* is well established and the ability to identify species using SEM and molecular methods is widely practiced, the question of the toxicity of *Gambierdiscus* species is the next, crucial data needed for a functional biogeography of the genus. Several laboratories have been working to isolate cells and grow cultures to concentrations high enough to allow extraction of ciguatoxin(s) and maitotoxin, the water soluble toxin in *Gambierdiscus* (Holland *et al.* 2012). As culture techniques have improved in tandem with improved assay and instrument sensitivity, the validation of functional assays like the N2A and receptor binding assay (RBA) by LC-MS is providing initial data about species-specific *Gambierdiscus* toxicity. It is the ability to overlay the relative toxicity onto species distribution and abundance maps that will provide the functional data layer that speaks to the risk of ciguatera fish poisoning. Despite this progress, the toxicity of specific *Gambierdiscus* species remains largely unknown.

Methods and Materials

In an effort to support subsequent CFP risk assessments, a literature search for species-specific *Gambierdiscus* cell toxicity was under-taken to summarize all data subsequent to the taxonomic revision completed by Litaker *et al*. in 2009. It is important to note when trying to compare the toxicity data, different extraction techniques as well as different analytical or functional assays were used in these studies. These data include the lipid soluble CTX and the water-soluble MTX fractions obtained from *Gambierdiscus* cell extracts.

Table 1. Toxicity of *Gambierdiscus* and *Fukuyo* species by method and location. CTX denotes ciguatoxin(s) and MTX denotes maitotoxin(s). RBA denotes receptor binding assay. LC-MM is liquid chromatography-mass spectrometry. The term ”Caribbean averaged” indicates that data from multiples isolates from different locations having the same toxicity patterns were combined. ND=Not detected and NA=Non acclimated strain.

Species	CTX	MTX	Method	Reference
G. australes				
French Polynesia RAV-92	Detected		RBA	Chinain *et al.* 2009
French Polynesia MUR-6	Detected		RBA	Chinain *et al.* 2009
French Polynesia MUR-14	Detected		RBA	Chinain *et al.* 2009
French PolynesiaTB-1	Detected		RBA	Chinain *et al.* 2009
French Polynesia RAI-5	NA/ND		RBA	Chinain *et al.* 2009
French Polynesia MG-4	ND		RBA	Chinain *et al.* 2009
Cook Islands CAWD216	ND	ND	LC-MS	Rhodes *et al.* 2010 & 2014
Cook Islands CAWD149	ND	Detected	LC-MS	Rhodes *et al.* 2010 & 2014
Hawaii W B Gam 3		Detected	Ca^{2+} Flux Assay	Lewis (ICHA2014)
Hawaii CAWD185	ND	Detected	LC-MS	Rhodes 2014
G. belizeanus				
St. Barthelemy STB-1	Detected		RBA	Chinain *et al.* 2009
Caribbean (averaged)		Detected	Haemolytic Assay	Holland *et al.* 2013
St. Barts CCMP 399	Detected	Detected	Ca^{2+} Flux Assay	Lewis (ICHA2014)
G. caribaeus				
Belize Gam 19	ND	ND	Ca^{2+} Flux Assay	Lewis (ICHA2014)
Hawaii Pat HI Jar 2 Gam2		Detected	Ca^{2+} Flux Assay	Lewis (ICHA2014)
G. carolinianus				
Caribbean (averaged)		Detected	Haemolytic Assay	Holland *et al.* 2013
Belize Dive 1 Gam 1		Detected	Ca^{2+} Flux Assay	Lewis (ICHA2014)
North Carolina Kenny 6	Detected	Detected	Ca^{2+} Flux Assay	Lewis (ICHA2014)
Hawaii Pat HI Jar3 Gam 9		Detected	Ca^{2+} Flux Assay	Lewis (ICHA2014)
G. carpenteri				
Australia (NSW)		MTX-like	Mouse Bioassay	Kohli *et al.* 2014
Belize GT4		Detected	Ca^{2+} Flux Assay	Lewis (ICHA2014)
Pat HI Jar7 Gam 11		Detected	Ca^{2+} Flux Assay	Lewis (ICHA2014)
G. excentricus				
Canary Islands VGO790	Detected	Detected	N2A	Fraga *et al.* 2011
Canary Islands VGO790	Detected	Detected	N2A	Fraga *et al.* 2011
Canary Islands VGO790	Detected	Detected	N2A	Fraga *et al.* 2011

Species	CTX	MTX	Method	Reference
G. pacificus				
French Polynesia HO91	ND		RBA	Chinain *et al.* 2009
French Polynesia MUR4	ND		RBA	Chinain *et al.* 2009
French Polynesia FP ET-2	ND		RBA	Chinain *et al.* 2009
Cook Islands CAWD213	ND	ND	LC-MS	Rhodes *et al.* 2010 & 2014
G. polynesiensis				
French Polynesia TB92	Detected		RBA	Chinain *et al.* 2009
French Polynesia RAI-1	Detected		RBA	Chinain *et al.* 2009
French Polynesia FP RG92	Detected		RBA	Chinain *et al.* 2009
French Polynesia FP MG-7	Detected		RBA	Chinain *et al.* 2009
Cook Islands CAWD212	Detected	ND	LC-MS	Rhodes *et al.* 2010 & 2014
TB-92	Detected	Detected	Mouse Bioassay	Chinain *et al.* 2009
G. ribotype 2				
Caribbean (averaged)		Detected	Haemolytic Assay	Holland *et al.* 2013
St. Maartens Gam 10	Detected	Detected	Ca^{2+} Flux Assay	Lewis (ICHA2014)
Puerto Rico mixed PR	Detected	Detected	Ca^{2+} Flux Assay	Lewis (ICHA2014)
F. ruetzleri				
Belize Gam1	Detected	Detected	Ca^{2+} Flux Assay	Lewis (ICHA2014)
G. scabrosus				
Japan	Detected	Detected	Mouse Bioassay	Nishimura *et al.* 2013
G. silvae				
Canary Islands	?	?		Fraga & Rodriguez 2014
G. toxicus				
Australian & Polynesian	Detected	Detected	Mouse Bioassay	Holmes *et al.* 1990
Tahiti FP GTT91	ND		RBA	Chinain *et al.* 2010
Tahiti FP HIT-0	ND		RBA	Chinain *et al.* 2010
Tahiti FP HIT-25	Detected		RBA	Chinain *et al.* 2010
Tahiti FP PAP1	Detected		RBA	Chinain *et al.* 2010
La Reunion FP REN-1	ND		RBA	Chinain *et al.* 2010
F. cf ***yasumotoi***				
New Zealand CAWD210, 211	ND	ND	LC-MS	Rhodes *et al.* 2010 & 2014

Results and Discussion

The results of this survey indicate that substantial work is still needed to provide a robust data set detailing the intra- and inter-specific variability in toxin content and toxin concentration required to map CFP risk. In the Pacific *G. polynesiensis* is consistently toxic with all six strains tested being positive for CTX and one of 2 strains being positive for MTX (Table 1; Chinain *et al.* 2009). *Gambierdiscus australes* tested positive for CTX in four of the nine of the strains tested and was

positive for MTX in three of four strains. Chinain *et al.* (2009) noted that one of these strains had not been acclimated prior to testing. Interestingly the type species, *G. toxicus* tested positive for CTX in only two of five strains. When Nishimura and his colleagues tested the newly described *G. scabrosus* using mouse bioassay, it was positive for both CTX and MTX activity. In the Caribbean two of three *Gambierdiscus* ribotype 2 strains tested positive in for CTX and all three for MTX. Only one strain of *F. ruetzleri* from the Caribbean has been tested for toxicity and it was positive for both CTX and MTX. In the eastern Atlantic all three strains of *G. excentricus* tested were positive for both CTX and MTX.

There is no record of *G. pacificus* being positive for either CTX or MTX in the three strains tested (Table 1). Neither CTX nor MTX was detected in the single strain of *F.* cf *yasumotoi* tested. This is ironic, in one sense, because of the pioneering work done by Professor Yasumoto on *Gambierdiscus /Fukuyo* and their toxins and yet, most fitting in that Professor Yasumoto is the most generous, selfless and helpful researcher in our field.

In two strains of *G. carpenteri* from the Pacific and one from the Caribbean none was positive for CTX but all three tested positive for MTX. One strain of *G. belizeanus* strain from the Pacific (Hawaii) tested positive for both CTX and MTX while a Caribbean strain (Belize) was non detect for both CTX and MTX. At this early stage of assessing the toxicity of *Gambierdiscus* and *Fukuyo* species, it is difficult to describe patterns in toxicity and there should not be too much emphasis placed on single isolate results. However, it is obvious that where we find *G. polynesiensis*, *G. australes* or *G. scabrosus* in the Pacific, *Gambierdiscus* ribotype 2 in the Caribbean and *G. excentricus* in the Atlantic there should be heightened concern for toxin transfer into the marine food web.

Even given the considerable amount of work summarized here, we still lack the critical database of *Gambierdiscus* species-specific toxicity required to assess CFP risk. This need is recognized, however, and many of the analytical obstacles have been overcome allowing ongoing efforts to provide this crucial information. As this field of BHAB research moves forward the vision of mapping *Gambierdiscus* species, species abundance and species-specific toxicity to assess CFP risk will become a reality.

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Ten years of *Didymosphenia geminata* in New Zealand

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Abstract

The stalked freshwater diatom *Didymosphenia geminata* has gained notoriety over the past 25 years as a significant nuisance algal species because it produces massive proliferations in oligotrophic fresh waters. The range of *D. geminata* blooms has been expanding since the 1990s, initially within its assumed natural Northern Hemisphere distribution range. In October 2004, *D. geminata* blooms were observed in a New Zealand river for the first time, prompting a government-backed response that highlighted the status of the proliferations as harmful algal blooms. Treatment of *D. geminata* as non-native in New Zealand reinforced a view that *D. geminata* blooms worldwide were caused by the human-mediated spread of an invasive variant. Ten years on, understanding of this diatom has changed. Research in New Zealand supports a view that *D. geminata* blooms are directly driven by low concentrations of inorganic phosphorus (P, < ~2 ppb), which lead to excessive stalk production when cell-division rates are P-limited. This mechanism is consistent with an environmental explanation (declining P in rivers) for the range expansion of blooms in the Northern Hemisphere. I review the history of *D. geminata* in New Zealand, including evidence supporting its assumed non-indigenous status, and research over the past 10 years into its impacts, biology and distribution.

Keywords: Didymosphenia geminata, non-native, oligotrophic rivers, phosphorus limitation, diatom stalks

Introduction

Harmful algal blooms (HABs) have been defined as including "all aquatic species that are known to produce toxins or to cause harm, directly or indirectly, to aquatic organisms or to terrestrial organisms associated with aquatic habitats or their products" (Landsberg 2002). Most HABs are toxin-producing marine or freshwater phyto-plankton but benthic freshwater taxa have recently been included. A majority of HABs can be attributed to increases in nutrient supply to the aquatic environment (Anderson *et al.* 2002). Here I present a New Zealand perspective on the freshwater benthic diatom species *Didymosphenia geminata* Lyngbye (M. Schmidt), commonly known as didymo or "rock snot" (Fig. 1). *D. geminata* proliferations arguably meet the general definition of HABs, but are non-toxic and form only in oligotrophic waters.

D. geminata has become notorious globally, particularly in the past 10 years because the species can produce massive proliferations in rivers. *D. geminata* blooms were first observed in New Zealand in October 2004 during routine monitoring in the Waiau River, Southland, South Island. The finding was alarming for two main reasons. First, the species had been reported as a nuisance alga in other locations, in particular Vancouver Island, Canada. Second, according to the literature, *D. geminata* was typically found in clean waters with low nutrient concentrations, raising fears of algal blooms in some of New Zealand's most valued pristine rivers.

Fig. 1. *D. geminata* mats against a backdrop of a bloom covering about 90% of the Mararoa River bed, Southland. (Photo: Southland Fish & Game).

D. geminata: a non-native, invasive species in New Zealand?

The initial concerns triggered an official response from the New Zealand government, led by Biosecurity New Zealand (now a section of the Ministry for Primary Industries, MPI). The first step was classification of *D. geminata* as an "unwanted organism". Such status required the organism to be non-native and several lines of evidence supported this view.

Of the thousands of diatom samples collected from rivers, lakes and fossil deposits in New Zealand for well over a century, there has been only one record of *D. geminata* – from a ditch near Wellington – and there is good reason to suspect that that record was a misidentification (Kilroy and Unwin 2011). Furthermore, the river where *D. geminata* blooms were discovered had been the subject of regular biological monitoring programmes (including detailed taxonomic analyses of periphyton) since the mid-1990s, with no reports of the species prior to 2004.

Following the discovery, blooms were traced upstream into the Mararoa River, which is a lake-fed tributary of the Waiau River. The blooms stopped at the Kiwi Burn, a popular area for river recreation (e.g., angling, rafting) about 60 km upstream of the site of first discovery. Upstream spread of *D. geminata* mats in the following 1–2 years was consistent with the view that *D. geminata* had not been present in the river prior to about 2004 or, at most, a few years earlier.

Unwanted organism status triggered a suite of studies in New Zealand investigating the biology, ecological, economic and social effects, potential and actual distribution, and control options (see www.biosecurity.govt.nz/didymo).

Biology and distribution

Didymosphenia geminata is remarkable in rivers because of the large size and robustness of its cells (Fig. 2). The stalks are exuded from an apical pore field, and, as in other stalked diatoms, comprise mainly carbohydrate. The cells form mats by first attaching to the substratum – generally rock surfaces in running freshwater. Once attached, the cells exude stalks, and then divide. Each daughter cell produces its own separate stalk (Fig. 2). The result after multiple cell divisions is a mass of stalk material with cells at the surface.

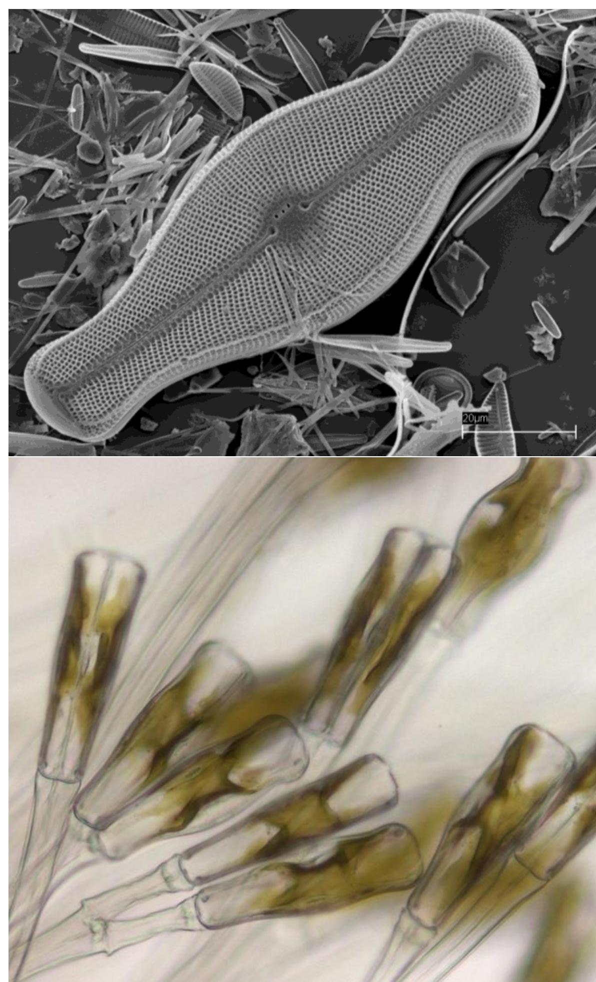

Fig. 2. *D. geminata*. Top. Scanning electron micrograph illustrating the large cell size relative to other diatom taxa. Bottom. Live cells attached to stalks, including cells in the process of division

How *D. geminata* arrived in New Zealand will never be known, but studies in 2004-5 showed that cells can remain viable for weeks in cool, damp conditions (www.biosecurity.govt.nz/files/pests/didymo/didymo-survival-dec-06-rev-may-07.pdf). Such survival allows for many potential human-facilitated transport scenarios, including cells trapped in sports equipment and felt-soled waders (Bothwell *et al.* 2009).

Since 2004, *D. geminata* has been detected in most major catchments in the South Island, and now dominates the periphyton assemblage in some rivers, although in many cases *D. geminata* has remained at low abundance. Circumstantial evidence for human-mediated spread of *D. geminata* in the South Island lies in the ability to link most records of the species at new locations to human activities such as angling, four-wheel driving, mountain biking, and kayaking (Kilroy and Unwin 2011).

An assessment of the likely spread of *D. geminata* within New Zealand suggested that the rivers most susceptible to blooms would be lake-fed or regulated systems, and rivers that regularly experience long periods of low flows (Kilroy *et al.* 2008). The assessment also concluded that the South Island was much more susceptible to proliferations than the North Island. This assessment has proved to be generally accurate. At the time of writing, no *D. geminata* cells had been detected in any North Island river. In addition it became clear that some South Island rivers were resistant, particularly spring-fed systems (Bothwell and Kilroy 2011).

Is *D. geminata* harmful?

Information available in 2004 indicated that *D. geminata* did not produce toxins and was not directly harmful to humans or animals. The principal concern was its ability to carpet up to 100% of river beds with unprecedented levels of periphyton biomass. It was difficult to imagine that such blooms would not affect other parts of the river ecosystem.

Subsequent studies in both New Zealand and overseas have documented effects on other algae (Kilroy *et al.* 2009, Gillis and Lavoie 2014) and have shown that the blooms are associated with shifts in macroinvertebrate community composition. Invertebrate assemblages dominated by mayfly, caddisfly and stonefly larvae (generally good food for fish) shift, in the presence of *D. geminata* blooms, to assemblages dominated by animals more tolerant of degraded habitat and water quality (worms, snails, midge larvae) (Gillis and Chalifour 2009, Kilroy *et al.* 2009, Larned and Kilroy 2014).

Effects on vertebrates were of most concern because of potential negative impacts on New Zealand's important recreational trout fishery and iconic native river birds. However, despite demonstration of changes in invertebrate food sources for fish, negative impacts on both salmonids and native fish taxa have proved difficult to demonstrate (e.g., Bickel and Closs 2008). Feared effects on native river birds did not eventuate, primarily because these birds tend to inhabit rivers with unstable flow regimes, which do not support persistent *D. geminata* blooms.

There have been economic and social effects and programmes are underway in several South Island rivers to control *D. geminata* blooms by manipulating flows (e.g., Lessard *et al.* 2013).

Global patterns

The natural geographical range of *D. geminata* is assumed to encompass cool-temperature boreal or mountainous regions throughout the Northern Hemisphere. The first record of the species was in the Faroe Islands in 1819, its early recognition attributable to its distinctive appearance. Blooms of *D. geminata* have been reported from Norway for over a century, as a natural phenomenon (Lindstrom and Skulberg 2008). Similarly, proliferations in rivers in England and Scotland are natural occurrences (Whitton *et al.* 2009).

The earliest reports of *novel* blooms were from Vancouver Island, starting in 1989, and their history has been described by Bothwell *et al.* (2009, 2014). Briefly, over several years, the blooms spread from river to river until much of the island was affected. The mystery for scientists was that the blooms were occurring in oligotrophic rivers, upstream of any human influence. Furthermore, there were historic records of *D. geminata* on Vancouver Island as far back as the late 19th Century, suggesting that environmental change might be driving the blooms. Environmental triggers were investigated, but no plausible link emerged.

In the 1990s novel *D. geminata* blooms were reported from other Northern Hemisphere locations: e.g., Iceland (Jonsson *et al.* 2000), and Poland (Kawecka and Sanecki 2003). However, arguably, the discovery of *D. geminata* in New Zealand in 2004 raised the profile of the blooms as a global issue. Because scientific opinion in New Zealand was that *D. geminata* was a new incursion of an invasive species, research efforts increased, as did suggestions that the blooms in other parts of the world (including Vancouver Island) were the result of the spread of an invasive genetic variant of the "original" species (Kirkwood *et al.* 2005, Bothwell *et al.* 2009).

Since 2004, blooms have been reported, as new phenomena, from many locations including North and South America, Europe and Asia (Bhatt *et al.* 2008, Blanco and Ector 2009, Montecino *et al.* 2014).

The "didymo paradox"

The most puzzling feature of *D. geminata* is its massive proliferations in oligotrophic waters. Initial assumptions were that *D. geminata* cells and/or stalks must possess some mechanism for acquiring phosphorus from low nutrient waters, which gave it a competitive advantage over other

algae (Ellwood and Whitton 2007, Sundareshwar *et al.* 2011). A different view emerged following research in New Zealand.

In 2008 a series of experiments was initiated by Dr Max Bothwell (Environment Canada). The research was conducted at a stream-side channel facility beside the Waitaki River, an oligotrophic, dam-controlled river draining to the east coast of the South Island. *D. geminata* blooms were first observed in the Waitaki River in early 2006.

The purpose of the research was to investigate the absence of *D. geminata* from spring-fed systems. Many such systems had clear pathways for colonisation but visible mats did not develop. The research partly resolved the issue of absence from spring-fed waters, and also clarified a plausible mechanism driving blooms in oligotrophic waters. Two results were key to the explanation.

1. *D. geminata* cell division rates were phosphorus-limited in Waitaki River water, despite prolific *D. geminata* biomass in the river; in contrast, cell division was stimulated in the more nutrient-rich spring water, despite the absence of visible mats in the spring creek (Bothwell and Kilroy 2010).
2. Stalk length in *D. geminata* was inversely related to cell division rates, and positively related to light intensity. In other words, when cell division rates were limited by low P in high light conditions promoting photosynthesis, stalk production increased (Kilroy and Bothwell 2011). The phenomenon of excessive carbohydrate (extracellular polymeric substance, EPS) production in nutrient limiting conditions is well known in diatoms, but has generally been reported as diffuse production of EPS in the marine environment (e.g., Staats *et al.* 2000).

A parallel field study carried out in 31 rivers throughout the South Island provided field data to corroborate the hypothesis that the blooms are driven by excessive stalk production when inorganic phosphorus concentrations are low enough to limit cell division rates (Kilroy and Bothwell 2012). In the surveys:

a) no blooms were observed at sites where mean inorganic phosphorus (dissolved [filterable] reactive P, DRP) exceeded about 2 ppb;
b) in rivers where *D. geminata* was present, cell division rates were inversely related to biomass (measured as a visual index);
c) cell division rates were positively correlated with mean DRP.

The third finding was important because it indicated that cell division rates were directly linked to DRP in the overlying water, a logical conclusion considering the location of most *D. geminata* cells at the surface of the mat.

The overall conclusion from the experiments and survey was that the proximate cause of *D. geminata* blooms is very low dissolved phosphorus, with the New Zealand data suggesting a threshold of about 2 ppb DRP (Bothwell *et al.* 2014).

An environmental explanation for the blooms

The realisation that low dissolved phosphorus concentrations may cause *D. geminata* blooms cast a new light on the origin of blooms in the Northern Hemisphere, first set out by Bothwell *et al.* (2014). At last there was a plausible explanation for the occurrence of blooms on Vancouver Island in the 1990s. Bothwell *et al.* (2014) stated:

> "In the case of Vancouver Island, it is now known that the onset of *D. geminata* blooms in the early 1990s followed the commencement of an island-wide annual urea-N fertilization silvicultural programme that was among the largest of its kind in North America …. Increased N inputs to landscapes will likely lead to increased P assimilation, reducing its availability for run-off into streams."

Subsequently, Taylor and Bothwell (2014) suggested that the global expansion in the geographical range of *D. geminata* blooms may be attributable to declining P in rivers to concentrations < 2 ppb. The fact that Taylor and Bothwell (2014) did not specifically exclude New Zealand from this hypothesis, raised the scenario of historical presence of *D. geminata* in New Zealand, undetected, with the blooms initiated some time prior to 2004 following declining DRP in some rivers.

To my knowledge, no suitable data on water nutrient concentrations exist for Vancouver Island rivers, which could be used to demonstrate declines in DRP coinciding with the forest fertilization programme mentioned by Bothwell *et al.* (2014). However, for New Zealand, data in the National River Water Quality Monitoring network (Davies-Colley *et al.* 2011) do not suggest any general pattern of declining P in oligotrophic rivers over the past 25 years. Mean annual DRP concentrations in rivers currrently affected by *D. geminata* blooms have been < 2 ppb since data

collection began in 1989 (author's unpublished analysis). If we accept the Taylor and Bothwell (2014) hypothesis, the lack of evidence for declines in DRP in the 15 years prior to 2004 actually supports the view that *D. geminata* was a new incursion into New Zealand. If the organism was already present, why did the blooms not appear until 2004?

However, because proving absence is impossible, the possibility that *D. geminata* was present in New Zealand long before 2004 cannot be wholly dismissed. We can only say that no convincing evidence to date indicates that the blooms in 2004 did not result from a recent incursion.

Non-detection in the North Island

Ten years after the first discovery of blooms, *D. geminata* has still not been detected in the North Island, despite ongoing surveillance (Kilroy and Unwin 2011). Non-detection could be due to an active campaign since 2004 to encourage river users to prevent spread by disinfecting items that had been in contact with river water (www.biosecurity.govt.nz/cleaning). However, the realisation that low DRP concentrations are a prerequisite for blooms provides another plausible explanation. DRP concentrations in most North Island rivers are higher than those in the South Island, and concentrations in rivers with *D. geminata* blooms are lower still (Fig. 3).

Inter-island differences in DRP do not satisfactorily explain the failure to find *D. geminata* even at low abundance in the North Island. Clearly, environmental factors other than DRP determine whether *D. geminata* can survive in a river, regardless of whether blooms form. One potential factor affecting distribution in New Zealand is temperature. *D. geminata* presence is associated with cool climates (Kumar *et al.* 2009). The South Island experiences cooler conditions than most of the North Island.

Other aspects of water chemistry may also be involved. For example, data from Norway indicate that *D. geminata* requires a certain concentration of calcium (Lindstrom and Skulberg 2008). Preliminary analyses suggest that water chemistry associated with *D. geminata* presence in the South Island may differ from that that in most North Isand rivers (author's unpublished analysis). However, laboratory studies have shown that *D. geminata* cells are capable of attaching and dividing in water from rivers in both islands, representing a wide range of water chemistry (Kuhajek *et al.* 2014). Research aimed at understanding the mechanisms controlling the distribution of *D. geminata* across catchments is ongoing.

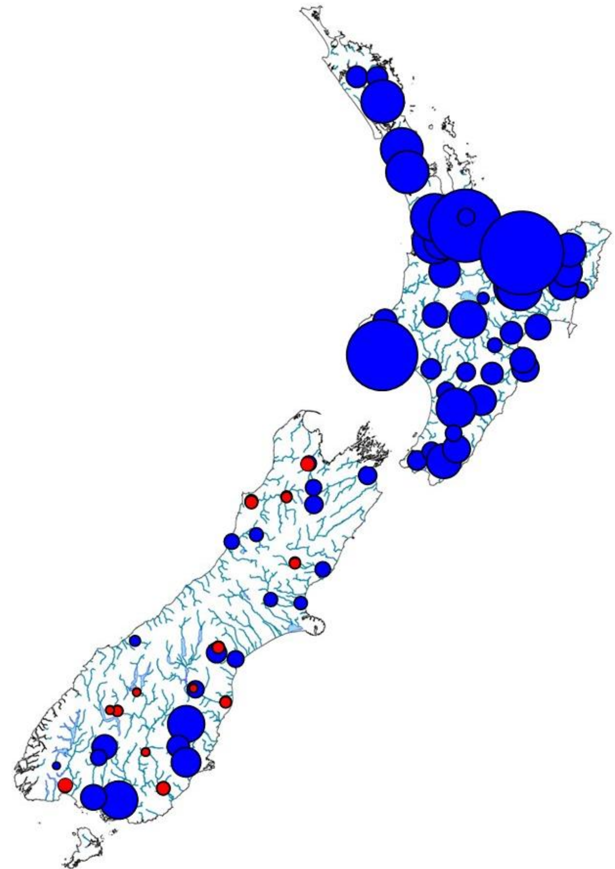

Fig. 3. Locations of the 77 sites in New Zealand's National River Water Quality Monitoring Network. Circle size indicates relative mean DRP concentration (2005-2013 data, range <1 – 67 ppb). Red circles are sites affected by *D. geminata* blooms.

Conclusions

When *D. geminata* blooms were first observed in New Zealand in 2004, the New Zealand response to this potentially harmful diatom was hampered by lack of information. Ten years on, understanding of the biology and impacts of *D. geminata* and its blooms has expanded considerably, partly because of the treatment of *D. geminata* as an invasive species in New Zealand. Research in New Zealand has demonstrated that *D. geminata* blooms in oligotrophic waters likely occur because of low phosphorus concentrations. Low P as the cause of *D. geminata* blooms is consistent with an environmental explanation for their spread in the Northern Hemisphere. Nevertheless, the weight of evidence currently suggests that *D. geminata* was a new introduction

to New Zealand in 2004 or thereabouts. The species appears to have spread and bloomed in rivers throughout the South Island because existing environmental conditions were ideal. Accordingly, the species is now established in many rivers and is here to stay. In contrast, North Island river environments generally do not favour blooms, although failure to detect *D. geminata* even at low abundance in the North Island is still not understood.

Acknowledgements

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Researchers as partners to the seafood industry and risk managers in developing appropriate risk management of harmful algal blooms

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Abstract

Harmful algal blooms can have devastating effects on wild harvest fisheries or aquaculture. Industry and regulators have a combined role in managing the risks to consumers and the security of future trade and market access. Recently Codex has increased its attention on non-traditional vectors for marine biotoxins. In Australia paralytic shellfish toxins were detected in abalone during a bloom of *Gymnodinium catenatum*, causing an industry estimated loss of A$1.38 million. Further to this an unprecedented bloom of *Alexandrium tamarense* impacted multiple fishing industries; with direct costs of A$6.3 million. Researchers were included at the outset of these events to address the significant knowledge gaps that hampered risk management: the difficulty in identifying and enumerating *A. tamarense*; the propensity of various tissues of vectors such as rock lobster and abalone to accumulate saxitoxins; the unknown consumption pattern of these tissues; a novel saxitoxin analogue; and the potential use of sentinel species for management. Underlying these knowledge gaps for the non-traditional vectors was the question of whether consumers were really at risk. These events highlight the role of risk analysis, and in particular of researchers in providing the science in a timely fashion to underpin appropriate risk management. The benefits of a partnership approach to managing food safety and trade and market access issues involving industry, regulators, and researchers, as facilitated by SafeFish in Australia, will be detailed.

Keywords: risk analysis, risk management, paralytic shellfish toxins, abalone, partnership approach

Introduction

The impacts of harmful algal blooms (HABs) on bivalve wild fisheries and aquaculture are well known and generally well managed in Australia. Risk management adheres to national guidelines and documented quality assurance systems. It is the shared responsibility of the seafood industry and government regulators. The scale of monitoring and management relies heavily on risk assessments to balance the need for consumer protection, with the facilitation of safe commercial activities. In some circumstances HABs are repetitive events and the risk management is relatively clear to implement, as information central to risk analysis is readily available through previous research. However, occassionally novel events occur where the science is not well known, and significant knowledge gaps exist. For these incidents, risk management necessarily takes a more precautionary approach; the balance shifts in order to cover the primary goal of protecting public health. This may impose unnecessary impost on industry, potentially making them unviable. In this instance, researchers become valuable partners to the seafood industry and regulators in order to fill the knowledge gaps, informing the risk analysis process and underpinning improved risk management. Two recent events in Australia involving marine biotoxins in non-traditional vectors have highlighted the importance of researchers being included in the risk analysis approach. In each case significant knowledge gaps hampered management decisions by regulators and the seafood industry and led to highly precautionary closures placing significant economic burden on the seafood industry. SafeFish was able to provide technical assistance to both industry and regulators during these HAB events, as well as rapidly facilitate research into the high priority knowledge gaps.

SafeFish

SafeFish is a technical food safety and trade and market access program in Australia developed with Australian Seafood Co-operative Research Centre funding to resolve technical food safety issues. It facilitates a process involving all stakeholders to define the seafood safety issues and research required, and to implement the

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appropriate independant studies. Stakeholders include Commonwealth and State regulators, industry, researchers, and fisheries research funding bodies.

SafeFish employs a process that regularly identifies and prioritises emerging seafood food safety issues, facilitates research into these, then feeds the results of the research back to stakeholders to allow trade negotiations or improved risk management. Through this process SafeFish develops technical advice that supports Australian stances at Codex and enables Australia to rapidly respond to seafood food safety issues.

International regulations for saxitoxin in non-traditional vectors

In 2006 the Codex Committee for Fish and Fisheries Products (CCFFP) began drafting a standard that included control of marine biotoxins in abalone. Traditionally marine biotoxins have been associated with bivalve shellfish (Ryder 2014). Accordingly, the Australian Food Standards Code regulates for marine biotoxins in bivalve molluscan shellfish, but does not extend this to other seafood products (FSANZ 2000). However saxitoxins have been found in other seafood products such as abalone and rock lobster (see below).

International regulations for marine biotoxins in seafood vary. With respect to paralytic shellfish toxins (PSTs), a maximum limit (ML) of 0.8 mg STX eq kg^{-1} is common, however, China applies this to all aquatic products, the US to all fish, Japan to shellfish, whilst the EU regulations cover molluscs, echinoderms, tunicates and marine gastropods. CCFFP standards include MLs for bivalve shellfish, and more recently for abalone.

PSTs in abalone

Australia has the world's largest wild-capture abalone fishery. The annual catch totaled around 4400 tonnes in 2012 (Skirtun 2013), representing approximately 25% of annual global harvest. The fishery is valued at A$220 million per annum, with >95% of product exported. Predominant markets are Hong Kong (54%), China (17%), Singapore (11%) and Japan (12%).

A significant portion of Australian wild abalone is exported as live, fresh or chilled product (average 51% over the last 5 years), with other major formats being canned, frozen meat, or frozen whole (28, 7 and 4% respectively).

In 2007 the EU imposed trade restrictions on Australian abalone exports worth approximately A$7 million per annum based on the draft CCFFP standard. The lack of knowledge of marine biotoxins in abalone in Australia was acknowledged by industry and regulators, and research into this issue was initiated.

Abalone are marine gastropods that graze on rocky reefs, predominantly found along Australia's southern coasts. They are generally not considered as high a risk for biotoxin accumulation as bivalve molluscs that filter feed. However, elevated levels of PST have been found in abalone in the past. Martinez *et al.* (1993) describe low levels of PST detected in abalone foot and viscera in Spanish abalone in 1991. In 1994 Japan detected PST in Spanish imported abalone (Nagashima *et al.* 1995). Further testing showed >23 times the bivalve regulatory level in abalone foot, and >3 times the bivalve regulatory level in abalone viscera (Bravo *et al.* 1996). No source for the PST was detected. In 1998 PST was detected at 1.5 times the bivalve regulatory level in abalone viscera in Australia, and just below the regulatory level in foot (Arnott 1998). Pitcher *et al.* (2001) describe PST at 20 times the bivalve regulatory level in South Africa in 1999, with the highest levels found in the foot.

Initial Risk Assessment

The initial research into PSTs in Australian wild abalone was conducted by the South Australian Research and Development Authority (SARDI). It consisted of three parts: tank studies looking at the propensity for abalone to accumulate PST, processing studies to look at the impact of canning, and an initial risk assessment.

In the tank studies green lip abalone were fed pellets similar to those used in commercial aquaculture, but containing high levels of PST from *Alexandrium minutum* cultures (Dowsett *et al.* 2011). The dosing regime was selected to approximate a worse-case scenario harmful algal bloom. The laboratory study showed that only low levels of PSTs were retained when abalone were fed high doses of the toxins over an extended period. The final levels of PSTs recorded in the edible portion of abalone were approximately 50

times lower than the maximum permissible limit of 0.8 mg STX eq kg^{-1} for PSTs in shellfish.

Two potential risk mitigation approaches were also assessed as part of the trial. It was shown that both scrubbing the pigment from the fringe of the abalone, a commercial process that occurs prior to canning placing abalone in clean seawater and feeding them an uncontaminated diet resulted in a decrease in toxin levels. The risk assessment predicted that the chance of PSP illness from the consumption of canned Australian abalone in the EU is 'Extremely Low'. - only one case of illness is expected to occur every 100 years (McLeod *et al.* 2010).

Through the SafeFish process the findings of the laboratory study and the risk assessment were provided in a package to assist officials to re-negotiate access for Australian abalone to the potentially lucrative EU market, enabling the market to be reopened in 2010. The findings were also presented to the CCFFP discussions on the development of the standard for abalone, in support of the Australian argument for a risk assessment approach rather than mandated monitoring for biotoxins.

Field Studies

Meanwhile, an extensive bloom of *Gymnodinium catenatum* was developing in the lower Huon Estuary in south-eastern Tasmania, and extending into the D'Entrecasteaux Channel. This organism blooms regularly in the area. The Tasmanian Department of Health and Human Services instigates closures of commercial shellfish farms and broadcasts public health warnings for recreational shellfishers when required. Illness is rare, but on this occasion a fish farm worker was hospitalised after missing the broadcast warnings (Turnbull *et al.* 2013).

The important point here is to understand why the risk management is so effective. It's because the research is well advanced. *G. catenatum* blooms have been investigated in detail in Tasmania and presented in many papers (Hallegraeff *et al.* 1988, Bolch and Hallegraeff 1990, Hallegraeff 1992, Hallegraeff *et al.* 1995, Butler *et al*. 2000, Todd 2001). The event started in early March 2011. *G. catenatum* cells peaked in late March, at around 450, 000 cells per litre. PST levels in the mussels followed closely, peaking at 16 mg STX eq kg^{-1} in late April. The bloom crashed soon after.

A discussion of all stakeholders determined a need and a mechanism for sampling the bloom with support from industry, who were aware of the dual risk – not only to public health, but also to trade and market access. A major component of the Tasmanian wild abalone harvest comes from the south-east corner impacted by the bloom– approximately 8% of Australia's wild abalone, or around A$18 million per year.

Pooled samples of abalone were taken from commercial and recreational zones in the D'Entrecasteaux Channel. The discovery of PST in the initial abalone samples led to several discussions between stakeholders, where SafeFish was able to add value to the risk management process by providing technical advice on sampling plans and analysis, and to help define and facilitate the immediate research needs to improve the on-going management.

In the following months, PSTs were found to be above the ML of 0.8 mg STX eq kg^{-1} in 11 of 156 viscera samples and in none of the 157 foot samples analysed. Concurrently sampled mussels showed 9 of 31 were above the ML. The maximum concentration recorded in abalone viscera was 1.74 mg STX eq kg^{-1}, in foot it was 0.52 mg STX eq kg^{-1} and in mussels it was 2.55 mg STX eq kg^{-1} (these figures are derived from established ratios of values from screen/confirmed results using an adapted Lawrence Method) .

Of particular concern was a novel saxitoxin analogue identified in potentially high levels in the abalone, preventing confident estimates of total PST levels. There was some evidence of low toxicity for this analogue, but none-the-less regulators took a precautionary approach whilst research was being undertaken.

Cawthron Institute in New Zealand purified and characterised the compound (Harwood et al. 2014), which was then sent to Mike Quilliam at the National Research Council in Canada, and a standard solution produced for future quantification. The compound was identified as deoxydecarbomyl saxitoxin and was determined by mouse bioassay to have a very low potentcy with a toxicity equivalency factor of 0.042.

Significantly the monitoring of PSTs in abalone during this bloom enabled the identification of the source of the PSTs. Samples of the gut tissue from abalone with high toxin levels, were examined for

toxic algae and genetic material. Although no remnants of toxic cells were found, genetic material from *G. catenatum* was detected in the viscera using species specific quantitative PCR (McLeod *et al.* in prep).

This confirmation opened many doors for management. Risk managers can now also monitor for the alga rather than the toxin alone, they can use information available on seasonality, bloom conditions, longevity of blooms etc, and they have the potential to combine monitoring with other fisheries for significant cost savings.

The field studies also provided data on the relative uptake and elimination rates of PSTs in mussels and different abalone tissues, which will aid in further risk management activities.

Again the information gained was able to inform the Australian position at future CCFFP discussions through the SafeFish process. The final Standard for Live Abalone and for Raw Fresh Chilled or Frozen Abalone for Direct Consumption or for Further Processing (FAO/WHO 2013), now requires "*the Competent Authority (using a Risk Assessment) to determine whether a risk exists in any geographical areas under its control and if so, put in necessary mechanisms to ensure that the part of the abalone to be consumed, meets with the marine biotoxin level in the Standard for Live and Raw Bivalve Molluscs (CODEX STAN292-2008)"*.

Provisional Risk Assessment

As SafeFish had been working closely with New Zealand colleagues during the standard development, the two countries combined to undertake risk assessments in order to both comply with the new Codex Standard to identify if we did or did not have a significant public health risk and to maintain market access.

In Australia we needed more information on the prevalence of biotoxins in abalone. The majority of the production comes from remote areas causing difficulties for sampling and transport. So we conducted a prevalence survey of harvested product (whole abalone) based on production (Malhi *et al.* 2014).

Samples were collected from 21 September 2012 to 1 December 2013 from all major abalone-fishing zones in each abalone-producing State, representing 80% of Australian wild caught abalone production. None of the 190 abalone sampled exceeded the bivalve regulatory levels for PST, AST, or DST. The resulting prevalence estimate for the survey period suggests that less than 1.6% of the commercially caught wild abalone population in Australia were contaminated with marine biotoxins at levels above the regulatory limit. ASTs were detected in the foot and viscera in very low levels. PSTs were also detected at very low levels in 17 samples of abalone foot tissue and 6 samples of abalone viscera. No DSTs were detected (Malhi *et al*. 2014).

For the risk assessment, this data was combined with the NRS survey of 51 abalone sampled between 2002-2004, where no toxins were detected, and the event monitoring described above, where PST where detected above regulatory levels in abalone viscera. The risk assessments examined the many different factors that impact on the risk: three critical aspects were identified. The conditions that must be met for abalone consumers to be at risk from any type of shellfish poisoning were firstly, high levels of the relevant toxin must be present in phytoplankton in the abalone growing area at the time of harvest or within a relatively short timeframe prior to harvest, secondly the abalone must be able to accumulate significant amounts of this toxin in the edible tissues, and finally, there is a requirement for individual human consumption of enough abalone to exceed the acute reference dose for that toxin by a margin significant enough to cause illness.

The risk assessment found that whilst all three requirements were possible, the risk of all occurring simultaneously were low to extremely low for most abalone products – the only exception being a low to moderate risk for abalone viscera sashimi. An extensive search of national databases and international literature failed to find evidence of any confirmed or probable cases related to the consumption of marine biotoxins via abalone, corroborating the finding that these conditions are rarely all met.

Benefits of a stakeholder approach

In this case study, the SafeFish approach of bringing all stakeholders together to prioritise and facilitate research, and then use the research outputs for direct input into technical trade negotiations, standard development and risk management has shown a number of advantages.

The science based approach has resulted in international acceptance of a risk based Codex standard. It has also protected market access to key international markets through the demonstration of low food safety risk. A direct benefit analysis showed that the investment by the abalone industry of approximately $600,000 or approximately 0.27% of the average annual export sales of Australian abalone resulted in avoidance of mandatory large scale biotoxin monitoring (A$20million + NZ$2-3 million p/a), and provided a number of alternative, cost effective risk management approaches.

Indirect benefits to the seafood industry in general came from the partnerships created, development of increased capabililty for analysis and research in marine biotoxins in Australia and New Zealand, and the creation of key research relationships between industry and researchers in these two countries.

The established SafeFish network of industry, governments, researchers was able to provide further value to all stakeholders a few months later during the October 2012 bloom of *Alexandrium tamarense* on the east coast of Australia (Campbell *et al.* 2013). This extensive offshore bloom of an alga not previously seen in the bivalve monitoring program in Tasmania effected the whole east coast. Multiple fisheries were impacted, including the high value rock lobster fishery.

As with the PSTs in abalone, this was a novel issue to Australia, involving significant knowledge gaps, and requiring risk managers to take a more precautionary approach. The direct cost of the fisheries closures was A$8.6 million. The indirect cost to the community was closer to A$23 million (Campbell *et al.* 2013).

Again the SafeFish platform and the approach of bringing all stakeholders together added significant value to the risk management process. By including researchers in the discussion SafeFish provided technical input to incident management, rapid initial risk analysis to underpin short term risk management, and helped to define the research questions and facilitate longer term research for ongoing improvements in risk management. Two major research projects have resulted from this process: a risk assessment for marine biotoxins in rock lobster to gain increased understanding of toxin dynamics in lobsters, assess the risk of PST's in rock lobster in HAB areas and provide options for management protocols; and a broader project aimed to provide improved understanding of Tasmanian harmful algal blooms and biotoxin events to support seafood risk management.

Conclusion

In summary, risk management is a difficult task. The more scientific knowledge available the better the mangement program is able to control and mitigate the risk. In cases where little information is available, the risk management is necessarily more precautionary as the protection of public health is the primary aim. Thus researchers have a vital role to play in underpinning appropriate risk management.

The approach taken by the SafeFish program in Australia to rapidly involve researchers in the risk analysis process has shown several advantages to all stakeholders. For industry the advantages are more cost effective risk management, increased capability in Australia, and resolution of trade and market access issues. For regulators the process has resulted in improved information for decision making, improved relationships with industry, faster uptake of new practices and a focus on priority issues. For researchers advantages have been grants, publications, on-going relationships , and the ability to solve issues of relevance to industry.

A significant advantage to Australia has been to lift capability, particularly in the area of seafood food safety. Improvements to analytical and research capabilities and capacity will have ongoing benefit. One of the biggest challenges is working together. Scientists approach a problem in a logical manner; we want to identify all the taxa, determine the processes and impacts, then look for appropriate management actions. But industry look at this from a different angle. They want the key burning questions answered first, with some interim management results to alleviate their worst issues. Balancing the need for immediate results with the longer term need to understand the system is a key component of working together to define the appropriate immediate research questions. Stakeholders need to work closely together with good communication to jointly determine the research priority list.

Acknowledgements

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Algal toxins and Toxicology

Novel toxins from the New Zealand red tide dinoflagellate *Karenia brevisulcata*

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Abstract

A red tide dinoflagellate bloomed in Wellington Harbour, New Zealand in 1998. This caused mass mortalities of fish and invertebrates within the harbour. Over 500 cases of human respiratory distress were also reported. The causative dinoflagellate was identified as the new species, *Karenia brevisulcata*, and its cell extract showed strong mouse lethality and cytotoxicity. The extract was partitioned between organic solvents and aqueous methanol under neutral and acidic conditions. From the neutral lipophilic fraction several novel polyether toxins were isolated and subsequently named brevisulcenals (KBT). The structure of KBT-F was determined to be a large polycyclic ether compound with 24 ether rings, including dihydrofuran, 13 hydroxy groups, and a 2-methylbut-2-enal side chain that is similar to those of gymnocins produced by *Karenia mikimotoi*. From the hydrophilic acidic fraction different toxins, named brevisulcatic acids (BSXs), were obtained. The structures of BSXs consist of nine contiguous ether rings and contain a 9-membered ether ring within the central portion of the molecule. BSX-4 showed cytotoxicity against neuro-2a cells analogous with brevetoxins and ciguatoxins.

Keywords: Karenia brevisulcata, Wellington Harbour, Polyether toxin, Brevisulcenal, Brevisulcatic acid

Introduction

A widespread bloom of a red tide dinoflagellate was observed in the central and southern east coast of the North Island of New Zealand in the late summer in 1997/98 (Chang 1999a). This HAB event led to massive kills of fish and invertebrates in Wellington Harbour. More than 500 cases of human respiratory distress were reported by harbour bystanders during the bloom with symptoms including a dry cough, severe sore throat, runny nose, skin and eye irritations, severe headaches and facial sunburn sensations. The new algal species, *Karenia brevisulcata* was identified as the causative dinoflagellate (Chang 1999b, 2001). This species is morphologically similar to *K. brevis* and *K. mikimotoi* (Chang 2011), which produce brevetoxins (Lin *et al*. 1981; Shimizu *et al*. 1986) and gymnocins (Satake *et al*. 2002, 2005; Tanaka *et al*. 2013), respectively. Structural features of these toxins are characterized by a trans-fused polycyclic ether ring framework with an unsaturated aldehyde terminus.

A cell extract from *K. brevisulcata* exhibited strong mouse lethality and cytotoxicity (Holland *et al*. 2012). It was partitioned between chloroform and aqueous methanol under neutral and acidic conditions, and each fraction contained different toxins. Lipophilic toxins in the neutral chloroform fraction demonstrated strong mouse lethality and cytotoxicity against P388 cells, and were named brevisulcenals (initially called karenia brevisulcata toxins (KBTs)). Their sodiated molecular cations in MALDI-MS were observed at over *m/z* 2000. These observation indicated that KBTs are large polycyclic ethers.

In addition to the KBTs, more water soluble polycyclic ether toxins were obtained from an acidic fraction. These were named brevisulcatic acids (BSXs) and had molecular weights of around *m/z* 900. BSXs were extracted with dichloromethane after adjustment of the crude extract to acidic conditions. Preliminary ESI-MS analysis, NMR evidence and toxicity data also indicated that BSXs were polycyclic ethers. In this paper, the structures of brevisulcenal-F (KBT-F) (Hamamoto *et al*. 2012) and brevisulcatic acids-1 (BSX-1) and -4 (BSX-4) (Suzuki *et al*. 2014) isolated from *K. brevisulcata* are described.

Material and Methods

Culture growth and harvesting of *Karenia brevisulcata*

Karenia brevisulcata (CAWD82) was collected from Wellington Harbour in 1998 and held in the Cawthron Institute Culture Collection of Microalgae (CICCM), Cawthron Institute, Nelson. Bulk cultures (150-250 L batches) were grown in

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12 L carboys using 100% GP + Se media under a 12/12 hour day/night timed cool white fluorescent lighting regime and 25 min aeration every 30 min. Starter culture (14-21 days old) was added to 100% GP + Se media at a ratio of 1:10 to 1:15. Cultures were maintained for up to 21 days. Aliquots of culture were assessed for cell numbers by inverted microscope. For ^{13}C enrichment, cultures were augmented at 0 and 7 days with $NaH^{13}CO_3$ (0.25g per 12 L). Production of toxins was assessed by liquid chromatography – mass spectrometry (LC–MS) using a described method (Harwood *et al*. 2014). Briefly, a 50 mL aliquot of culture was extracted with Strata-X SPE cartridge (60 mg, Phenomenex Inc., CA), washed with Milli Q water and 20% methanol, and eluted with methanol or methanol followed by acetone (3 mL each). Toxins were extracted from mature cultures using Diaion HP20 resin. The pre-washed resin was packed in a polypropylene column. *K. brevisulcata* cultures were transferred to a 200 L barrel and cells were lysed by addition of acetone to 7% v/v. The cultures were settled for one hour and diluted with reversed osmosis purified water to 5% v/v acetone before pumping at 0.3 L/min through a filter system followed by the HP20 resin column. The column was then washed with water and the HP20 resin was transferred to a 2 L flask. Toxins were recovered by soaking the resin with AR grade acetone (1L) and decanting. The combined acetone extract was rotary evaporated to produce a dried crude extract.

Isolation of KBTs

The crude HP20 extract was dissolved in methanol and diluted to 55% v/v with pH 7.2 100 mM phosphate buffer. The solution was partitioned with chloroform and the combined chloroform fraction containing neutral toxins was evaporated. Brevisulcenals were isolated from the neutral chloroform fractions of bulk cultures (1450 L) by column chromatography using a diol cartridge with stepwise elution (ethyl acetate to methanol) and guided by cytotoxicity assay. Final purification was by two stages of preparative HPLC (4.6×250mm Develosil C30-UG-5, Nomura Chemical Co., Japan) with isocratic elution (90% $MeOH/H_2O$ followed by 85% $MeOH/H_2O$) and guided by UV absorbance at 230 nm.

Isolation of BSXs

After the 55% MeOH fraction was adjusted to pH 4.0, BSXs were extracted with dichloromethane. After evaporation of dichloromethane in vacuo the extract was resupended in water:MeOH (1:1) and then subjected to solid phase extraction on Strata-X resin using stepwise elution using various water:MeOH combinations (1:1, 4:6, 3:7, 2:8, 1:9, 0:1). The 100% MeOH fraction, which contained the BSXs, was subjected to further chromatographic separation on a Develosil ODS-UG-5 (4.6×250mm) column with a gradient elution method using MeOH/water (80% $MeOH/H_2O$ to 100% $MeOH/H_2O$ over 40 min). The flow rate was 1.0 mL/min and the column oven was set to 35°C to ensure stable retention times. Elution of BSXs was monitored using LC–MS .

Results and Discussion

Isolation of KBT-F

From 1450 L cultures, 3.1 mg of KBT-F was isolated. Similarly, 1.3 mg of ^{13}C enriched KBT-F was isolated from 250 L cultures with added ^{13}C-$NaHCO_3$.

Structure of KBT-F

KBT-F showed a UV absorption maximum at 227 nm. This result indicated that KBT-F possessed a conjugated system in the molecule. A sodium adduct ion peak in the positive ion MALDI-MS spectrum was observed at *m/z* 2076. The MALDI-SpiralTOF accurate mass and ^{1}H and ^{13}C NMR spectra indicated that the molecular formula of KBT-F was $C_{107}H_{160}O_{38}$ ($[M+Na]^+$ 2076.0476, calcd. 2076.0480).

The structure of KBT-F (Fig. 1) was determined by detailed analysis of NMR and MALDI-SpiralTOF–TOF spectra. KBT-F has 24 ether rings including an unusual dihydrofuran, 13 hydroxyl groups, 13 methyl groups, and a 2-methylbut-2-enal terminus. KBT-F contains 17 contiguous ether rings A-Q, which is the longest of known polycyclic ethers. The long contiguous ether ring assembly with the 2-methylbut-2-enal terminus is similar to those of gymnocins. Unlike the brevetoxins, KBT-F does not have unsaturated middle sized ether rings in the molecule. The molecule consists of two parts, one is the rigid ether ring assembly, rings A-Q and the other is a flexible acyclic part and the short ether ring assembly rings S-X. The majority of the 13 hydroxyl groups reside in the flexible part of the molecule from C-60 to C-94, while all the angular methyls reside on the rigid ring assembly A-Q.

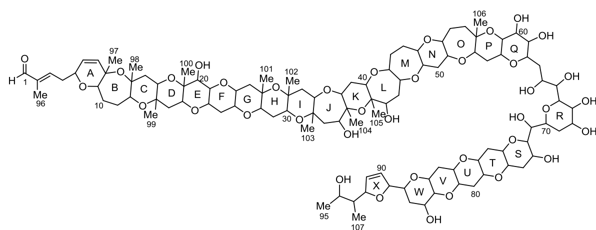

Fig. 1. Structure of brevisulcenal-F (KBT-F).

Biological activities of KBT-F

Acute toxicity of KBT-F was determined to be 0.032 mg/kg, which is more toxic than that reported for many brevetoxin analogues. KBT-F also showed strong cytotoxicity against mouse leukemia P388 cells at 2.7 nM (Hamamoto *et al.* 2012).

Isolation of BSX-1 and BSX-4

From 150 L culture media, 0.17 mg of BSX-1 (**2**) and 0.62 mg of BSX-4 (**3**) were obtained by repeated chromatographies on a reverse phase column. To facilitate ^{13}C related NMR measurements, ^{13}C labelled BSX-1 (1.2 mg) and BSX-4 (1.1 mg) were isolated from cultures grown in media enriched with ^{13}C $NaHCO_3$.

Structures of BSX-1 and BSX-4

No UV maximum above 210 nm indicated that BSX-1 and BSX-4 did not have conjugated bond systems. The HR ESI-MS gave the molecular formula of BSX-1 as $C_{49}H_{72}O_{16}$ and that of BSX-4 as $C_{49}H_{70}O_{15}$. These molecular formulas are similar to that of brevetoxin A ($C_{49}H_{70}O_{13}$) (Shimizu *et al.* 1986). Detailed analysis of NMR spectra indicated that a 5-membered lactone structure was assigned to BSX-4, which is analogous to brevetoxin A (Fig. 2). Structural interconversion under acidic and basic conditions, and the observed difference between the molecular formulas of BSX-1 and BSX-4 being H_2O (18 Da), suggested that BSX-4 contained a lactone and BSX-1 had a carboxylic acid. The NMR spectra also suggested that the structures of BSX-1 and BSX-4 were identical except for this A ring. Analysis of the NMR spectra measured at 25˚C gave some signals that were not assignable because they were extremely broad, and some missing NMR signals around the rings E to G in the middle portions of BSX-1 and BSX-4. This was presumed to be caused by slow conformational perturbation of a 9-membered ether ring as observed in brevetoxin A and ciguatoxins (Murata *et al.* 1990). NMR spectra of BSX-1 and BSX-4 were remeasured at low temperature (-20°C), as was used to solve this problem for the ciguatoxins. This resulted in appearance of new signals and sharpening of broad peaks around rings D, E and G allowing the structures of BSX-1 and BSX-4 to be elucidated. The proton chemical shift of H-4 for BSX-1 was at δ 3.25 ppm in CD_3OD and that for BSX-4 was at δ 4.28 ppm in pyridine-d_5. These indicated that the position of the lactone bond was determined at C4 and BSX-4 had the lactone as for ring A in brevetoxin A, while BSX-1 was a seco acid analogue of BSX-4 (Fig. 2).

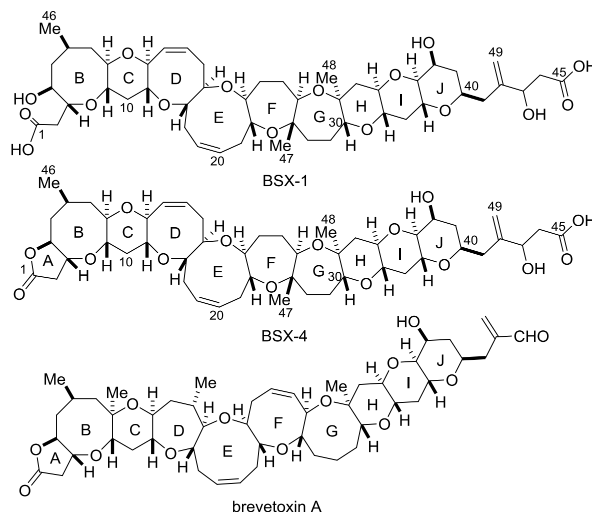

Fig. 2. Structures of brevisulcatic acid-1 (BSX-1), brevisulcatic acid-4 (BSX-4) and brevetoxin A.

Strcutural confiramation of BSX-4 was also accomplished by a MALDI-SpiralTOF–TOF experiment. BSX-4 did not give rise to an intense negative ion that was suitable as a precursor ion for MS/MS experiments because the carboxylic acid is a weak charge site for MALDI ionisation. Therefore, BSX-4 was converted to a taurine derivative by amidation to give a sulfonic acid as a strong charge site on the terminus. An intense precursor ion of the taurine derivative was observed at *m/z* 1004.5 and the MALDI-SpiralTOF-TOF produced prominent product ions generated by bond cleavages at the characteristic sites observed for other polycyclic ethers. As a result, the structure of BSX-4 was confirmed as shown in Figure 2. BSX-4 has 9 contiguously fused ether rings (8/6/8/9/7/7/6/6/6-membered rings), 2 hydroxyl and 3 methyl groups, and a γ-lactone and a carboxyl group at both termini.

Cytotoxicity of BSX-1 and BSX-4

In the neuroblastoma cell assay with the presence of veratridine and ouabain, BSX-4 gave a cytotoxicity EC_{50} of 24.5 nM, while that for BSX-

1 was 390 nM (Truman *et al*. 2007; Suzuki *et al*. 2014). The 15-fold higher cytotoxicity of BSX-4 than that for BSX-1 indicates that the γ-lactone (ring A) plays a significant role in the cytotoxicity of BSX-4. Without addition of veratridine and ouabain, BSX-4 did not show cytotoxicity against neuro-2a and the cytotoxicity was inhibited by addition of saxitoxin, indicating that the mode of action is activation of the voltage sensitive sodium channels, similar to that of brevetoxins and ciguatoxins.

In conclusion, two new ladder-frame polyether toxin groups, the brevisulcenals (KBTs) and brevisulcatic acids (BSXs), were isolated from the dinoflagellate *Karenia brevisulcata*, and were likely repsonsible for the unprecented toxic effects observed during the original bloom. Interestingly, the long contiguous ether ring assembly with the 2-methylbut-2-enal terminus in KBT-F is reminiscent of gymnocins from *K. mikimotoi*, while the γ-latone, the 9-membered ether ring, and rings A-C and H-J of BSX-4 resemble those of brevetoxin A from *K. brevis*.

K. brevisulcata cell extracts exhibited cytotoxicity against a human cancer cell line (P388), acute mouse lethality, hemolysis (Holland et al. 2012) and also ichthyotoxicity (Shi et al. 2012). Brevisulcenals are thought to be responsible for cytotoxicity against P388 cells, mouse lethality and observed haemolytic affects. However, the causative toxins of the potent ichthyotoxicity have not been identified. BSX-4 has a similar structure and activity to brevetoxin A. This raises the possibility that BSXs may be responsible for some of the ichthyotoxicity observed for *K. brevisulcata*. These types of studies will be conducted in the future.

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Development of two novel UPLC-MS/MS methods for the analysis of maitotoxin from micro-algal cultures

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Abstract

Two fast, sensitive and selective ultra-performance liquid chromatography mass spectrometry (UPLC-MS/MS) methods have been developed to rapidly screen micro-algal cultures of the toxic benthic dinoflagellate *Gambierdiscus* for maitotoxin production. The first method monitors the intact toxin and can be used directly on micro-algal culture extracts. Novel aspects of this approach include use of alkaline mobile phase for chromatographic separation and monitoring the MTX di-anion using a pseudo-MRM transition. The second method has been developed to detect a specific oxidation product of maitotoxin that is generated from a simple on-column oxidation procedure. This approach is similar to that recently described for palytoxin and exploits the known oxidation chemistry of periodic acid, which can cleave carbon-carbon bonds between vicinal diol groups.

Keywords: Gambierdiscus, maitotoxin, polyether marine toxin, UPLC-MS/MS

Introduction

The marine benthic dinoflagellete *Gambierdiscus* produces many polyether toxins including ciguatoxins (Murata et al., 1989), maitotoxin (Holmes et al., 1990; Yasumoto et al., 1977), gambieric acids (Nagai et al., 1992), gambierol (Satake et al., 1993), gambieroxide (Watanabe et al., 2013), gambierone (Rodríguez et al., 2015). Maitotoxin (MTX) is a water-soluble cyclic polyether ladder marine toxin that was first described in the mid-1970s. The complex molecular structure and stereochemistry was subsequently determined. MTX represents the largest natural non-biopolymer compound known with a with a molecular formula of $C_{164}H_{256}O_{68}S_2Na_2$ (Fig 1; MW 3425.9 g mol^{-1}) and is extremely toxic with an LD_{50} of 0.05 µg kg^{-1} via intraperitoneal injection (Murata et al., 1993).

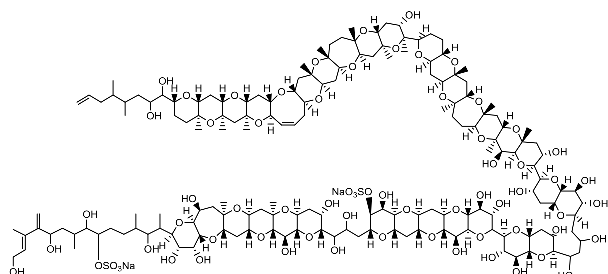


Fig 1. Molecular structure of maitotoxin (MTX).

The role of MTX in ciguatera fish poisoning remains unclear and there remains a need for sensitive methods of analysis to monitor its production, distribution, uptake and metabolism in the environment. Various detection methods, both analytical (Lewis et al., 1994) and functional (Caillaud et al., 2010), have been developed for MTX, although they have struggled for sensitivity (HPLC with UV detection) and specificity (mouse bioassay and N2a assay).

Mass spectrometric methods are powerful, particularly when coupled with liquid chromatography (LC-MS). They have been used for the qualitative and quantitative analysis of various marine toxin classes, including lipophilic shellfish toxins (McNabb et al., 2005) and paralytic shellfish toxins (Boundy et al., 2015). The analysis of MTX by LC-MS is challenging due to the lack of reference material and generation of complex mass spectra, with the ionisation spread across multiple charge states and the large ^{13}C isotope contributions. These issues typically lead to ambiguities in identification with low sensitivity and specificity for trace detection.

In this article we describe two novel analytical approaches that have overcome the various technical challenges associated with the analysis of MTX by LC-MS/MS. One approach monitors the intact toxin and the other a specific oxidation cleavage product generated using an on-column oxidation procedure. These methods can be used to screen *Gambierdiscus* cultures and fish for the presence of MTX.

Material and Methods

Sample preparation

Gambierdiscus culture samples (5-50 mL fresh aliquot) were centrifuged at 3000 x g for 5 min to pellet cells and the supernatant was discarded. The cell pellet was extracted twice with 5 mL 1:1 methanol:water by ultrasonicating for 2 min in a 59 kHz bath (model 160HT, Soniclean Pty, Australia), centrifuging at 3000 x g for 5 min and then pooling the resulting supernatant. Dilutions of MTX standard were prepared in 1:1 methanol:water.

LC-MS instrumentation

A Waters Xevo TQ-S triple quadrupole mass spectrometer (Xevo TQ-S) coupled to a Waters Acquity UPLC i-Class with flow through needle sample manager was used for all analyses.

Intact MTX analysis method

For screening, methanol-extracted algal extracts were diluted into 1:1 methanol:water (v/v) in glass auto-sampler vials and directly injected for LC-MS/MS analysis. Extracts were centrifuged prior to analysis. Chromatographic separation was achieved using an Acquity BEH phenyl 1.7 µm, 100 x 2.1 mm column held at 40 °C. The column was eluted at 0.5 mL min^{-1} with Milli-Q water (A) and acetonitrile (B) mobile phases, each containing 0.2% ammonium hydroxide (25% ammonia solution; v/v). The initial solvent composition was 90% A for 0.5 min, followed by a linear gradient to 50% B at 3.5 min, stepped up to 70% B at 3.6 min and a linear gradient to 100% B between 3.6 and 7.0 min. The column was re-equilibrated with 90% A between 7.0 and 8.0 min. The autosampler rack chamber was maintained at 10 °C and the injection volume was 2 µL. For MS monitoring, the electrospray ionization source was operated in negative-ion mode at 150 °C, capillary 3 kV, cone 50 V, nitrogen gas desolvation 1000 L h^{-1} (600 °C), cone gas 150 L h^{-1}, and the collision cell was operated with 0.15 mL min^{-1} argon. A pseudo multiple reaction monitoring (MRM) transition *m/z* 1689.6/1689.6 (CE 70 eV) was acquired for the intact MTX di-anion with a 50 ms dwell time.

On-column oxidative cleavage method

For determination of the MTX-specific oxidation cleavage product from micro-algal cultures, an on-column oxidation step was required prior to LC-MS/MS analysis. A 60 mg Strata-X SPE cartridge was pre-conditioned with 3 mL methanol and then 3 mL 1:1 methanol:water (v/v) containing 0.1% formic acid. MTX standard, or culture extract (0.5-4 mL), was then loaded in 1:1 methanol:water (v/v) containing 0.1% formic acid and washed with 2 mL milli-Q water to remove methanol. The sample was then oxidized on-the-column by passing 2 mL of 50 mM periodic acid under gravity at ca 1 mL min^{-1}. For elution, 1.8 mL of acetonitrile–water (1:4 v/v) containing 20 mM ammonium acetate was used. This was determined to be the optimum elution volume. The eluant was transferred into a glass vial for LC-MS/MS analysis. Chromatographic separation was achieved using a Thermo Hypersil Gold aQ 1.9 µm, 50 x 2.1 mm column held at 40 °C. The column was eluted at 0.30 mL min^{-1} with Milli-Q water (A) and acetonitrile (B) mobile phases, each containing 0.1% formic acid (v/v). The initial solvent composition was 100% A for 0.5 min, followed by a linear gradient to 60% B at 2.0 min, and then flushing with 95% B between 2.5 and 3.0 min. The column was re-equilibrated with 100% A between 3.0 and 4.0 min. The autosampler rack chamber was maintained at 10 °C and the injection volume was 10 µL. For MS monitoring, the same conditions were used as for the intact MTX method. For quantitative analysis, a MRM transition *m/z* 971.5/96.9 (CE 25 eV) was acquired with a 50 ms dwell time. A confirmatory MRM transition (*m/z* 971.5/953.5), was also monitored. For all LC-MS/MS analyses the peak areas of the target analytes were integrated and sample concentrations calculated from linear calibration curves generated from a MTX standard (see acknowledgments). TargetLynx software was used for the analysis.

Results and Discussion

Intact MTX analysis method

Development of a LC-MS/MS method for the detection of intact MTX allows rapid, sensitive screening of potential MTX-producing micro-algal cultures. Quantitation is possible, although appropriate steps are required to ensure method performance is suitable. MTX elutes in first linear portion of the gradient (Fig 2.). Due to the volatility of ammonia, fresh mobile phases were essential to maintain reproducible retention times. The step gradient described allows monitoring of several ciguatoxins within the same analysis, albeit with later retention times (4-8 min; data not presented). If MTX is the only analyte targeted then the inlet method can be shortened.

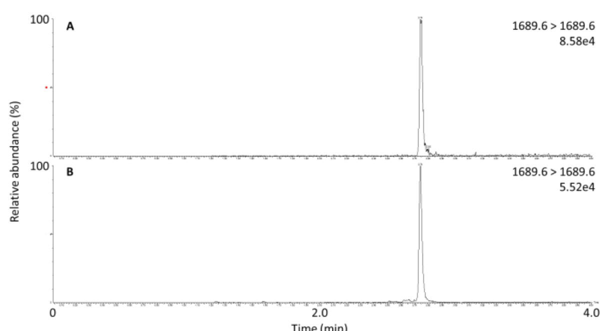


Fig 2. MTX MRM chromatograms (parent→parent) acquired for A) a 25 ng mL^{-1} MTX standard and B) an extract generated from a *Gambierdiscus australes* culture (CAWD149).

An alkaline mobile phase was used as it gave favourable chromatographic separation and resulted in improved negative electrospray ionisation sensitivity. The di-anion $[M-2H]^{2-}$ of MTX (*m/z* 1689.6) was monitored because the singly charged anion was outside the mass range of the mass spectrometer used and the tri-anion $[M-3H]^{3-}$, although observed, (*m/z* 1126.1), was less abundant (Fig 3.).

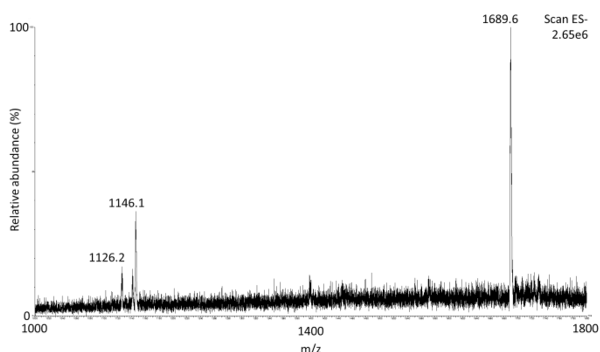


Fig 3. Full mass spectrum (*m/z* 1000-1800) of a MTX infusion showing the abundant di-anion (*m/z* 1689.6). Other masses represent the tri-anion (*m/z* 1126.2) and a mass consistent with a MTX acetic acid adduct (*m/z* 1146.1). Spectra generated using Micromass Quattro Ultima instrument.

The *m/z* 1689.6 ion was the most abundant and incorporates two ^{13}C atoms. For MRM analysis the di-anion was remarkably stable within the collision cell and only partially fragmented, even with high collision energies. This proved advatageous for monitoring the parent→parent transition (pseudo MRM), as applying a high CE (70 eV) substantially reduced background noise and improved the signal-to-noise of the MTX peak. The intact MTX method has good sensitivity with a LOD of approximately 1 ng mL^{-1}.

On-column MTX oxidative cleavage method

This new method has been used to monitor culture extracts for the presence of MTX (Fig 4.). The approach used is similar to that described by our research team for analysis of palytoxin (Selwood et al., 2012), another large polyether marine toxin. Monitoring smaller, specific substructures of target analytes is useful as it overcomes many of the challenges encountered when analysing large molecules, like MTX. Also, by performing a SPE procedure allows removal of matrix interferences (especially for complex matrices like fish extracts) and can improve method sensitivity by concentrating the target analyte.

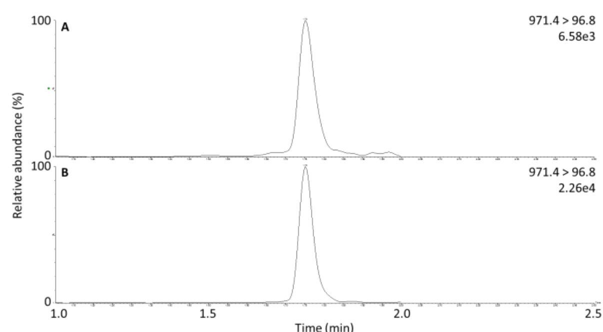
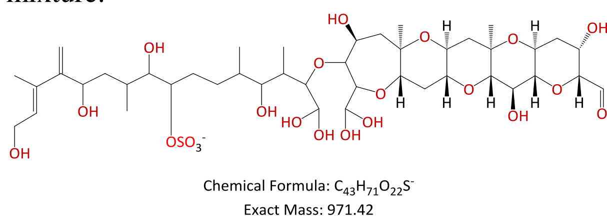


Fig 4. MRM chromatograms acquired for MTX from A) a MTX standard (20 ng mL^{-1}) and B) an extract of *Gambierdiscus australes* (CAWD149).

Periodic acid cleaves C-C bonds between vicinal diols via oxidation to yield carbonyl groups as either aldehydes or ketones, depending on substitution. The molecular structure of MTX contains 6 vicinal diols, 4 in ether rings and 2 in alkyl chains. Treatment with periodic acid results in 3 fragments being generated. Of these, one is not suitable for LC-MS/MS analysis as it is too small and does not contain an ionisable group, one contains an ionisable group but is not suitable as it is too large for MS analysis, and one that regarded as suitable for LC-MS/MS analysis. The predicted structure for the suitable fragment contains 3 aldehydes, 2 from the oxidation of the diol in an ether ring and the other from cleavage of the backbone of the molecule. Analysis of a MTX standard treated directly with periodic acid gave several oxidation products, with the most intense observed at *m/z* 971.6 (Structure 1). This ion is consistent with the mass of the proposed MTX oxidation product, with addition of two water molecules (+36 amu). This observation can be explained by hydration of two of the aldehyde groups formed during the oxidation procedure. However, we have not determined which of the aldehydes are hydrated. An ion that was consistent

with the oxidation product containing three aldehydes was observed (*m/z* 935.6), albeit at a much lower abundance. Other ions observed could be rationalised by the addition of just one water molecule, or the formation of hemiacetals due to the presence of methanol in the reaction mixture.



Structure 1. Proposed molecular structure of MTX oxidation product used for monitoring purposes.

For quantitative purposes it was not desirable to have multiple oxidation products, as it spreads the available charge across multiple species and reduces signal. To limit hemiacetal formation, acetonitrile was used as the organic solvent rather than methanol (acetonitrile does not react with aldehydes). To monitor the selected oxidation product, MRM transitions were established from a product ion scan experiments performed at varying collision energies. This showed several abundant water loss ions and also a strong signal at *m/z* 96.9, which represents bisulphate anion through loss of the sulphate group (Fig 5.). The on-column oxidation method showed linear calibration (5-50 ng mL^{-1}) and was determined to be at least as sensitive as the intact MTX method (<1 ng mL^{-1}). Matrix interferences can severely compromise electrospray ionisation. The on-column oxidation SPE procedure described for MTX removes many co-extractives that are present in complex sample types, such as fish. This makes it possible for fish extracts to be monitored for the presence of MTX and also increases the sensitivity of the method through analyte concentration. The method was used on fish (*Pagrus auratus*) samples generated as part of a research project investigating MTX uptake (Kohli et al., 2014). Samples were prepared as described and spike recoveries at the 0.45 mg kg^{-1} level were as follows; 83% for liver, 114% for intestine and 118% for flesh.

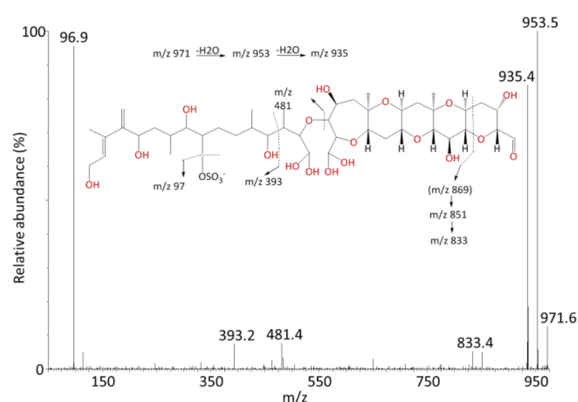


Fig 5. Product ion scan (*m/z* 971.6), and likely cleavage sites, of the selected MTX oxidation product used for monitoring purposes.

Acknowledgements

The MTX standard used was a gift from Prof Takeshi Yasumoto (Japan Food Research Laboratories, Tokyo).

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Ostreopsis cf. *ovata* from the Mediterranean Sea. Variability in toxin profiles and structural elucidation of unknowns through LC-HRMS^n

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Abstract

Over the last decade, massive blooms of *O.* cf. *ovata* have occurred in the whole Mediterranean Sea with consequent negative impacts on human health mainly by skin contact and inhalation of toxic aerosols. Previous studies demonstrated that *O.* cf. *ovata* produces a number of palytoxin congeners, that we named ovatoxins (OVTXs). In this study, a new LC-HRMS^n (n=1-4) approach, which provides complete chromatographic separation and unambigous identification of all the OVTXs known so far, has been used to characterize toxin profiles of 50 *O.* cf. *ovata* strains collected at different sites of the Mediterranean basin. All the analyzed strains turned out to produce ovatoxins and toxin profiles may be quite different from both qualitative and quantivative standpoints. In addition, the analysis of the fragmentation pattern contained in HRMS^n spectra of each compound allowed to higlight the region (or even the sites) where structural difference between OVTX-a, -b, -c, –d and -e occur.

Keywords: Ostreopsis cf. *ovata*, LC-HRMS^n, ovatoxins, Mediterranean Sea

Introduction

Blooms of *Ostreopsis* spp., once confined to tropical and subtropical areas, have recently spread to more temperate regions, including the Mediterranean and the Southern-Atlantic coasts of Europe. Over the last decade, massive blooms of *O.* cf. *ovata* have increased in frequency with consequent negative impacts on benthic communities as well as on human health mainly by skin contact and inhalation of toxic aerosols. The most alarming toxic events associated with *Ostreopsis* blooms occurred in Italy in 2005, in Spain in 2004 and 2006, and in France in the 2006−2009 period (Ciminiello *et al.* 2014). During these outbreaks, people exposed to marine aerosols during recreational or working activities required medical attention due to respiratory distress and in some cases intensive care hospitilisation.

Liquid chromatography high resolution MS (LC-HRMS) studies characterized *O.* cf. *ovata* as the producer of several congeners of palytoxin (PLTX, $C_{129}H_{223}N_3O_{54}$), one of the most potent non-protein marine toxins so far known (Deeds and Schwartz 2010). We named these molecules ovatoxins (OVTXs) and found that they differ structurally very little from the parent compound (Ciminiello *et al.* 2010; Ciminiello *et al.* 2012a). OVTX-a, the major component of the *O.* cf. *ovata* toxin profile, has been recently isolated and structurally elucidated (Ciminiello *et al.* 2012b). Only LC-HRMS data are available for OVTX-b, -c, -d, -e, and -f as they have not been isolated yet.

PLTX and OVTXs share a characteristic electrospray (ESI) ionization behaviour and chromatographic properties. In more detail, positive ESI MS spectrum of each compound contains a mixture of singly, doubly, and triply charged ions with the calcium adduct $[M+H+Ca]^{3+}$ being the base peak. In addition, in HRMS^n spectra these molecules fragment at many sites of their alyphatic backbone resulting in many diagnostic fragment ions. These ions provide insights into their complex structures even when OVTXs are contained at nanomolar concentration in crude extracts. As a result LC-HRMS^n (n = 1-4) spectra of OVTXs represent a kind of fingerprint of this group of molecules and can be used for their identification and stuctural characterization (Ciminiello *et al.* 2012a,c). Chromatographically, OVTXs may overlap under inappropriate chromatographic conditions and this may lead to misassignement of their moleculare formula. Very recently we optimized LC conditions for an efficient separation of palytoxin congeners which

A. Lincoln MacKenzie [Ed]. *Marine and Freshwater Harmful Algae 2014*. Proceedings of the 16th International Conference on Harmful. Algae. Cawthron Institute, Nelson, New Zealand and the International Society for the Study of Harmful Algae (ISSHA)

permits their unambigous LC-HRMS determination (Ciminiello *et al*. 2015).

The developed LC-HRMS approach (Ciminiello *et al*. 2015) has been used to characterize toxin profiles of 50 *O*. cf. *ovata* strains collected at different geographical sites in the Mediterranean basin. All the analyzed strains produced OVTXs and this study showed that toxin profiles may be quite different from both qualitative and quantivative standpoints. In addition, the analysis of HRMS^n spectra of each compound, allowed to higlight the region (or even the sites) of structural difference between OVTX-a, -b, -c, -d and -e.

Material and Methods

O. cf. *ovata* strains were collected at 8 sites of the Italian coasts (Ancona, Alghero, Genova, Pisa, Porto Romano, Bari, Trieste, Taormina) and at one site of the French Mediterranean coast (Villefranche sur Mer). A minimum of 6 to 9 samples for each location were collected. PCR assay using two primer set targeting ITS-5.8S regions were carried out for species-specific identification of *O*. cf. *ovata* (Battocchi *et al.* 2010). Clonal cultures were extablished and maintained in F/4 medium (Casabianca *et al.* 2014) and cells were collected at the end of the exponential phase for chemical analyses. Cell pellets (1-3 x 10^6 cells) of each strain were extracted with a methanol/water (1:1) solution and 0.2% acetic acid and sonicated for 3 min in pulse mode, while cooling in an ice bath, centrifuged at 3000g for 30 min. The supernatant was decanted and the pellet was washed twice more with methanol/water (1:1, v/v) and 0.2% acetic acid. The extracts were combined and directly analyzed by LC-HRMS^n (n = 1-3) using a hybrid linear ion trap LTQ Orbitrap XL Fourier transform mass spectrometer (ThermoFisher, USA) equipped with an ESI ION MAX source and a LC binary system (Agilent 1100, USA). A Poroshell 120 EC-C18, 2.7 µm, 2.1×100 mm column was used. Mobile phase was A=H_2O, 30 mmol L^{-1} acetic acid, and B=95% MeCN–H_2O, 30 mmol L^{-1} acetic acid. The following gradient elution was used: 28% B at t=0, 29% B at t=5, 30% B at t=15, 100% B at t=16, and hold for 5 min; re-equilibration time was 14 min. Flow was 0.2 mL min^{-1} and injection volume was 5 µL. HR full MS spectra (positive mode) were recorded in the range *m/z* 800−1400 at a resolution of 60.000, according to Ciminiello *et al.* 2015. Extracted ion chromatograms were obtained from the HR full MS data by selecting the $[M+H+Ca]^{3+}$ ion of each toxin (5 ppm mass tolerance). The chromatographic peaks were identified by comparing their retention times and associated HR full MS spectra to those of known OVTXs and isobaric PLTX contained in a reference *O.* cf. *ovata* extract previously characterized (Guerrini *et al*. 2010) and analyzed under the same experimental conditions. Peak areas were used in quantitative analyses using the calibration curve of PLTX standard (Wako Chemicals, Germany) as reference. Analytical standards for OVTXs are not available, thus quantification was estimated by assuming that these compounds would have the same molar response as PLTX.

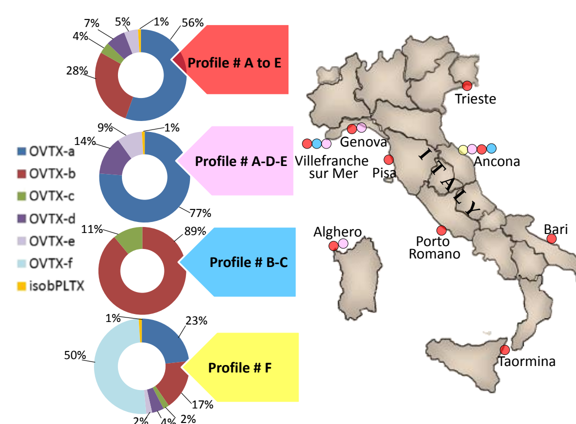

Fig. 1. Toxin profiles of *O*. cf. *ovata* including relative percentage of each toxin and distribution at collection sites. Profile # A to E (red dots), profile# A-D-E (pink dots), profile # B-C (light blue dots), Profile # F (yellow dots).

LC-HR collision induced dissociation (CID) MS^n analyses were performed for further confirmation of toxin identity. The $[M+H+Ca]^{3+}$ ion of each compound was used as precursor under a collision energy of 35%, an activation Q of 0.250, and an activation time of 30 ms. Data interpretation was done according to Ciminiello *et al.* 2012c.

Results and Discussion

Fifty cultured strains of *O.* cf. *ovata* collected along the Italian and the French Mediterranean coasts were extracted and analysed by LC-HRMS. All the strains showed to produce toxins. Figure 1 shows the relative percentage of each toxin and distribution of toxin profiles at collection sites.

Four types of toxin profiles were identified. ***Profile # A to E.*** 73% of the strains produced OVTX-a as the dominant toxin, followed by

OVTX-b, OVTX-d/-e, OVTX-c and isobaric PLTX, listed in decreasing order of concentration. In only 1 strain from Ancona, besides the above toxins, trace amounts of OVTX-f were detected.

Profile # A-D-E: 21% of the strains produced only OVTX-a, -d/-e and isobaric PLTX.

Profile # B-C: 4% of the analyzed strains produced only OVTX-b and –c.

Profile # F: this unique toxin profile was found in one strain from Ancona (Marche, Italy), and presented OVTX-f as the dominant toxin followed by OVTX-a,-b, -d/-e, -c and isobaric PLTX. LC-MS/MS analysis according to Selwood *et al.* 2012revealed that OVTX-b found in this strain was actually an isobaric OVTX-b (T. Harwood, personal communication).

LC-HRMS^n spectra of OVTX-a to -f were obtained for each sample and compared to OVTXs' contained in the reference sample (Guerrini *et al*. 2010). This allowed confirmation of the identity of individual toxins. Structural insights into OVTX-b to –e contained in the reference extract were gained through interpretation of their fragmentation patterns in LC-HRMS^n spectra. As a way of example the LC-HRMS^2 spectrum of OVTX-b is shown (Fig. 2). PLTX was used as template for data interpretation according to Ciminiello et al. (2012c).

Compared to PLTX, all OVTXs presented an additional hydroxyl group at C42 and lacked 2 hydroxyl groups, one at C17 and the other one in the region between C53 and C78. OVTX-a, -b, and -f lacked also the hydroxyl group at C44. In the small region between C6'-C8', OVTX-b and -c presented 2 additional methylene groups while OVTX-e presented an additional hydroxyl group. Finally OVTX-f presents two additional methylenes in the region between C95-C102 (Fig. 3). Several new OVTXs were also identified although they accounted for less than 1% of the total toxin content. Work is on-going on their identification.

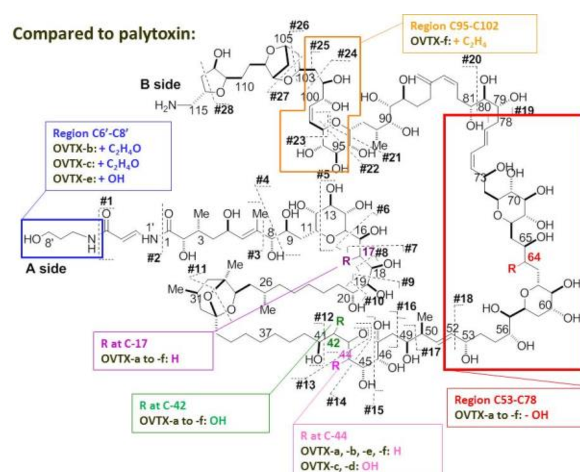

Fig. 3. Structural differences between PLTX (R at C17= OH; R at C42 = H, R at C44 = OH, R at C64 = OH) and OVTX-a to –f.

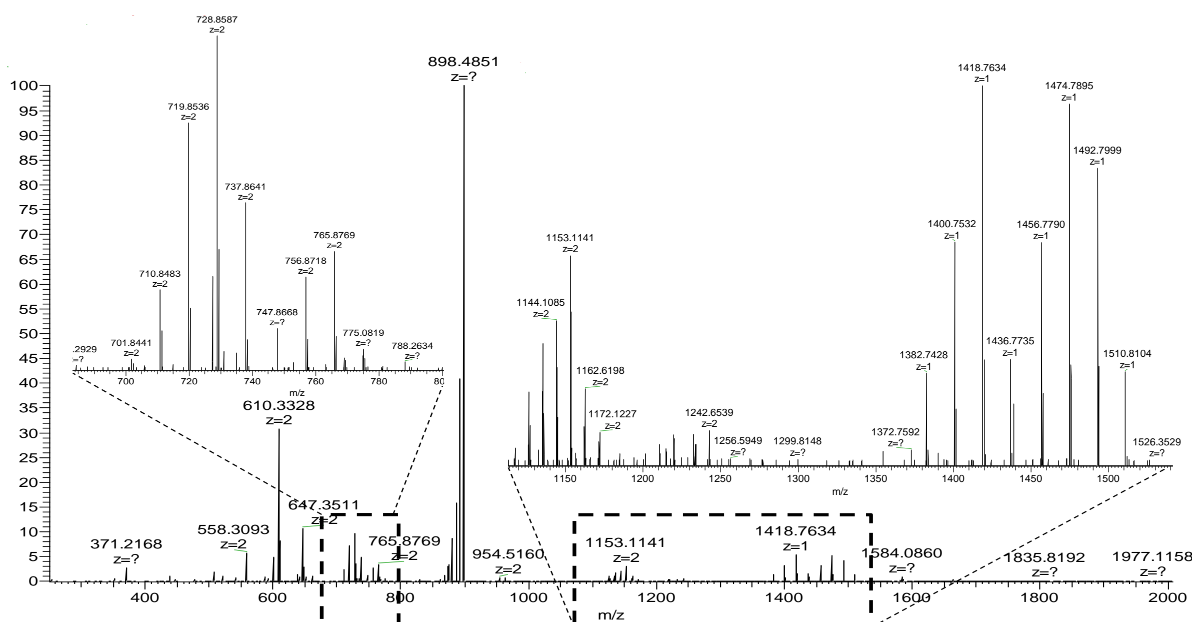

Fig. 2. LCHRMS^2 spectrum of OVTX-b.

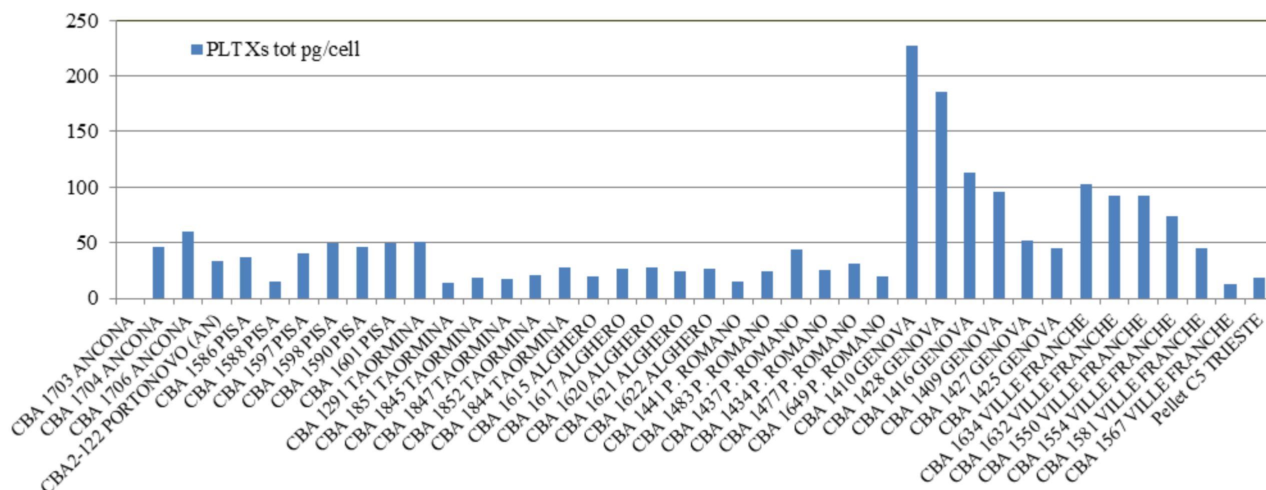

Fig. 3. Total toxin content (pg $cell^{-1}$) of the analyzed *O.* cf.*ovata* strains.

Quantitative analyses revealed that on a per cell basis, total toxin contents of the analyzed strains were in the range 5 to 230 pg/cells (Fig. 4). Most of the strains, however, produced OVTXs at levels of 5-60 pg/cell. Very high toxin contents (60-230) were shown only by strains collected in Genoa and Villefranche sur Mer sites (North western Mediterranean coasts). These sites are geographically very close and are actually the sites where the most alarming toxic outbreaks for number of people and severity of symptoms occurred so far. Since culturing condition, although do not affect toxin profile, might affect toxin productivity, in our study all the *O.* cf. *ovata* strains were cultured under the same nutrient, temperature and salinity conditions, collected on the same growth phase, and counted according to the same methodology. Thus, our quantitative results suggest that strains from the North-Western Mediterranean area are likely the most productive in the Mediterranean basin. This finding is in good agreement with the high toxin productivity data reported by Garcia-Altares *et al.* (2015) for the Spanish strains (Ebro Delta coasts) and by Brissard *et al.* (2014) for the French strains (Villefranche sur Mer). These data trigger some questions on the reasons why toxin profiles and contents of *O.* cf *ovata* may be so different and on the toxins to monitor in environmental and seafood samples.

Acknowledgements

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The regulation of toxicity in laboratory cultures of *Heterosigma akashiwo* from Puget Sound, Washington

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Abstract

Extensive finfish mortalities due to blooms of the raphidophycean flagellate *Heterosigma akashiwo*, (Y. Hada) Y. Hada ex Y. Hara et M. Chihara is problematic for many commercial aquaculture operations located in Puget Sound, Washington, USA. Decreased salinity has been linked to both bloom formation and increased toxicity for this raphidophyte, however it is unknown if toxicity increases at salinities below 20, or if there is a combinatorial effect with temperature. Using a non-axenic strain of *H. akashiwo* isolated from Puget Sound, cultures were exposed to a combination of three salinities (32, 20 and 10) and five temperature (14.7, 18.4, 21.4, 24.4 and 27.8 ºC) conditions. Our results indicate that the toxicity of unialgal cultures of *H. akashiwo* increased as salinity decreased from 32 to 10. Furthermore, at salinities of 20 and 10, neither temperature nor specific growth rate were correlated with ichthyotoxicity. However at the salinity of 32, both temperature and specific growth rate were inversely proportional to toxicity, which is likely due to the effect of contamination by an unidentified species of *Skeletonema* in those cultures. Competition with the *Skeletonema* sp. resulted in an ichthyotoxic response from *H. akashiwo* that was greater than the stress caused by salinity alone.

Keywords: Heterosigma akashiwo, salinity, temperature, ichthyotoxicity

Introduction

In estuaries, variations in phytoplankton growth and community composition are linked to the intrinsic differences in salinity, temperature, and the availability of light and nutrients. An estuarine system with an exceptionally complex mosaic of environmental factors is the Salish Sea – the inland waters of southwestern British Columbia, Canada, and northwestern Washington, USA. This region has a long history of blooms of the fish-killing flagellate *Heterosigma akashiwo*. The complexity of environmental conditions in the Salish Sea is generated through the different patterns and magnitudes of supply of oceanic water from the Pacific through the Strait of Juan de Fuca, and the contribution of freshwater from major river systems, in particular the Fraser River which flows into the Strait of Georgia. Blooms of *H. akashiwo* have coincided with increased river flows from snowmelt in Northern Puget Sound, with exceptionally large blooms detrimental to fish aquaculture operations occurring during those years with seasonally earlier and larger river flows from the Fraser River (Rensel *et al*. 2010). The ichthyotoxicity of *H. akashiwo* has been demonstrated to increase as salinity decreases from 40 to 20 (Haque and Onoue 2002). However, even with this laboratory study, our knowledge is still woefully incomplete with respect to the relationship between salinity and ichthyotoxicity. First, their study only examined salinity conditions above 20, whereas salinities above and below this are observed both seasonally and spatially in the Salish Sea (Sutherland *et al*. 2011). Second, only non-native strains to the Salish Sea were tested, and the relative toxicity of *H. akashiwo* varies markedly among strains (Cochlan *et al.* 2014). Therefore, it is unclear if toxicity is regulated by environmental factors specific to a geographical region or is solely a function of strain variability. Third, ichthyotoxicity was estimated from the measured concentration of brevetoxin-like compounds, and quantified using HPLC analysis (Khan *et al.* 1997). This is problematic given that these compounds from *H. akashiwo* have since been shown not to alter the sodium balance of isolated cell lines as seen for brevetoxins, but instead dramatically change calcium homeostasis, indicative that the putative

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toxin is a unique bioactive metabolite (Twiner *et al.* 2005).

The current study describes laboratory batch experiments designed to test the effects of salinity on a non-axenic strain of *H. akashiwo*, isolated from the Salish Sea. The objectives of this research were: 1) to determine if ichthyotoxicity is a function of salinity for a native strain to the Salish Sea, 2) to determine if a combinatorial effect exists between salinity and temperature on ichthyotoxicity, and 3) to evaluate the relationship between ichthyotoxicity and the specific growth rate of *H. akashiwo*.

Material and Methods

Culturing: H. akashiwo strain NWFSC-513, isolated in 2010 from Clam Bay, WA, USA was used throughout this study. Non-axenic cultures of NWFSC-513 were grown in 0.2-µm filtered (Polycap 150 TC; Whatman) coastal seawater, and enriched with a modified ESNW medium (Harrison *et al.* 1980; Berges *et al.* 2001). Modifications to the ESNW enrichment stocks were as outlined by Auro and Cochlan (2013), with the exception that reduced concentrations of inorganic N and P (50 and 5 µM, respectively) were used to prevent inorganic carbon limitation resulting from biological activity (e.g., Howard *et al.* 2007). Additionally, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot H_2O$ and Na_2SeO_3 were added at final concentrations of 3.93×10^{-8} M, 2.42×10^{-6} M and 6.36×10^{-9} M, respectively.

To produce growth media at three salinities (32, 20, 10), a single batch of natural seawater was collected from East Sound, WA (salinity = 32) using 10-L Niskin bottles (General Oceanics), and the lower salinities (20 and 10) were created by dilution with Milli-Q® ultra-pure water (18.2 MΩ · cm; EMD Millipore). To adjust for the dissolved inorganic carbon (DIC) removed during dilution, additional $NaHCO_3$ was added to the 20 and 10 salinity treatments to achieve a final concentration of 2.07×10^{-3} M. Temperature and light conditions were regulated within a custom-made light box that generated a temperature gradient through an aluminum block, with temperatures ranging from 14.7 to 27.8 ºC. Cells were exposed to an average PPFD of 350 ± 112 µmol photons$\cdot m^{-2} \cdot s^{-1}$ on a 14:10 h light: dark cycle. This PPFD, measured with a 4-π collector (QSL-100 Quantum scalar irradiance meter; Biospherical Instruments Inc.), is saturating for the growth of NWFSC-513 (Ikeda, 2014).

Batch cultures were grown in duplicate (n = 2), 50-mL borosilicate (Pyrex®) culturing tubes, equipped with Nalgene® polyethylene caps. After a 4-day acclimation period to the various combinations of temperature and salinity, the cultures were transferred to fresh media, and the quantification period initiated with quadruplicate cultures for each temperature/salinity treatments. Daily subsamples were collected aseptically from each of the culture tubes for determination of cell abundance by flow cytometry (BD Accuri C6 flow cytometer; BD Bioscience). Specific cell growth rates were calculated from least-squares linear regression analysis of the exponential phase of cell growth, determined from plots of the natural log of cell abundance versus time. Due to the large volume requirements for ichthyotoxicity analysis, samples were only collected during the stationary growth phase to ensure that the cells were nutrient depleted, and therefore most likely to be toxic (Cochlan *et al.* 2014).

Ichthyotoxin Analysis: H. akashiwo cells were harvested by filtration onto 25-mm glass-fiber filters (Whatman GF/F), followed by storage at -20 ºC. The intracellular ichthyotoxin was then extracted by adding 4 mL of methanol (LC-MS grade) to 12-mL glass tubes containing the filtered *H. akashiwo* cells. Cells were removed from the filters by gentle agitation for 2 min (or until the cells were re-suspended), and then centrifuged at *ca.* 1,700 g for 15 min. The supernatant was removed with a disposable glass pipette, and placed into a 4-mL amber speed vac vial. The ichthyotoxin contained in the supernatant was concentrated using a centrifugal evaporator (Oligo Prep OP120 concentrator) to remove the methanol, until 1 mL of extract remained. This extract was blow-dried using ultra-high purity nitrogen gas (grade 4.8), followed by re-suspension in 1 mL of L-15 (Leibovitz 1963) to a final density of 5.0×10^5 cells$\cdot mL^{-1}$. This solution was vortexed for 1 min, and stored at -20 ºC until analysis. Cellular ichthyotoxicity was analyzed following a modified version of Dorantes-Aranda *et al.* (2011), which measures the viability of a rainbow trout gill cell line (RTgill-W1) after exposure to *H. akashiwo* extracts. Ichthyotoxin extracts were thawed and sonicated to break up the cellular matter, before centrifuging (3,500 g) for 10 min. One hundred µL of the extract was added to a flat bottom, 96-well microplate

(Corning 353072) containing the live rainbow trout gill cell line (ATCC® CRL-2523™) at 90% confluence, which was then incubated for 6 h in the dark at 19 °C. After incubation, the ichthyotoxin extract was removed and replaced with 100 μL of L-15ex medium containing 5% (v/v) AlamarBlue® dye (Invitrogen DAL1025), and the plated samples were incubated for an additional 2 h. Gill cell viability was determined by the change in fluorescence (excitation: 540 nm, emission: 600 nm) due to the digestion of AlamarBlue® in *H. akashiwo* cell extract samples relative to control samples (without cells) measured using a Biotek Synergy 2 microplate reader.

Results and Discussion

The greatest toxicities were found in cultures of *H. akashiwo* grown at the salinity of 32 for the temperatures of 14.7, 18.4 and 21.4 ºC, followed by the cultures at salinities of 10 and 20 over the entire temperature range (Fig. 1). Cellular toxicity did not vary as a function of temperature or specific growth rate for cultures grown at salinities of 20 or 10. However for cultures grown at the salinity of 32, cellular toxicity decreased at the two highest temperatures (24.4 and 27.8 °C). Unlike any of the other salinity treatments, cultures grown at this salinity at the three lower temperatures (14.7, 18.4 and 21.4 ºC) were contaminated with an unidentified species of the diatom genus *Skeletonema*. Unialgal conditions of *H. akashiwo* were maintained at the two higher temperatures at this salinity, and in the other treatments. A unialgal culture of the *Skeletonema* sp. was examined for toxicity using the gill cell assay, and toxicity was found to be below the level of detection, indicating the non-toxic nature of these *Skeletonema* cells (B. Bill per. com.), thus these cells did not contribute directly to the toxicity measured here. For unialgal cultures of *H. akashiwo,* the cellular toxicity generally decreased with increasing salinity. The average toxicity of the unialgal cultures measured at the two highest temperatures (24.4 and 27.8 ºC) varied inversely with salinity, and cells grown at the salinity of 32 were 1.5- and 2.1-fold less toxic than cells grown at salinities of 20 and 10, respectively.

This pattern of reduced toxicity at lower salinities follows the pattern previously reported for other laboratory studies of ichthyotoxic raphidophytes, including *Chattonella* spp. (Haque and Onoue 2001), *Fibrocapsa japonica* (de Boer *et al.* 2004) and *H. akashiwo* (Haque and Onoue 2002). Our results also support the observation of large detrimental blooms of *H. akashiwo* during times of reduced salinity conditions in the Salish Sea, resulting from earlier and larger outflows of the Fraser River (Rensel *et al.* 2010).

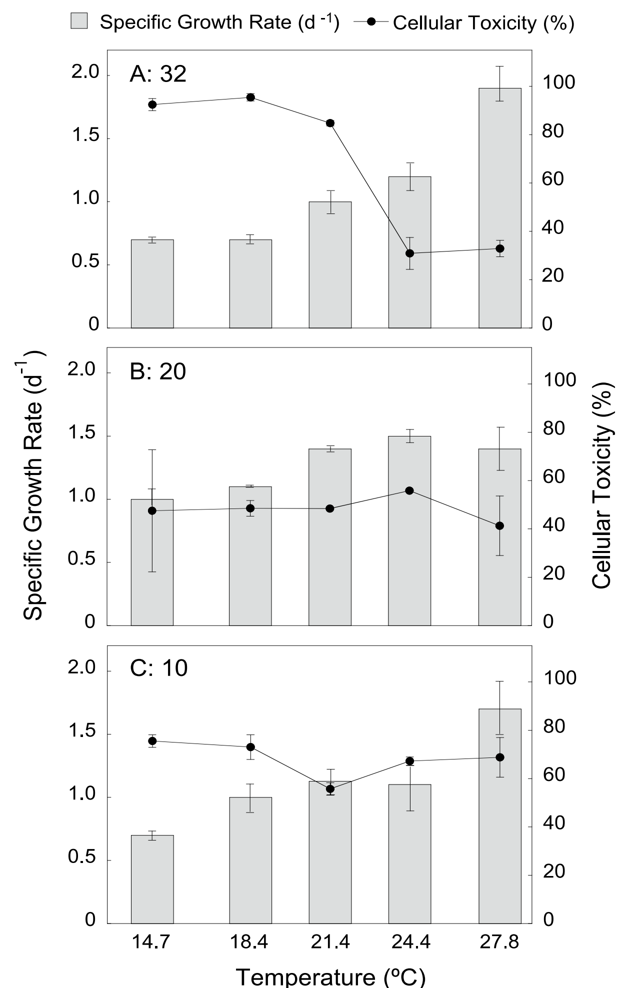

Fig. 1. Comparison of specific growth rates and cellular toxicity for *H. akashiwo* cultures grown at salinities of 32 (A), 20 (B), and 10 (C). Specific growth rates are reported as the mean ± 1 SD (n = 4) of quadruplicate cultures measured during the exponential phase of growth, and toxicity values are the mean ± range of duplicates (n = 2) during stationary phase of growth.

Neither temperature nor the increase in the specific growth rate affected cellular toxicity for cultures grown at salinities of 20 and 10 (Fig. 1B and C). For cultures grown at a salinity of 32 (Fig. 1A), increased temperature resulted in corresponding increases in specific growth rate, but these cultures (14.7, 18.4, 21.4 °C) were contaminated with an unidentified species of the diatom *Skeletonema.* Given that the fastest

growing cultures occurred at the two highest temperatures (24.4, 27.8 °C) and were unialgal, suggests that contamination, not temperature or growth rate, was responsible for the enhanced ichthyotoxicity observed in our study. Due to the close occurrence of *H. akashiwo* and *Skeletonema* blooms during spring and summer months, several studies (e.g., Pratt 1966; Yamasaki *et al.* 2007; Xu *et al.* 2010) have suggested that *H. akashiwo* uses an allelopathic compound to establish population dominance over *Skeletonema* species. In these studies, the initial cell densities were found to be a critical factor in the establishment of population dominance, which may explain the inverse relationship observed in the present study between the specific growth rate of *H. akashiwo* and ichthyotoxity for cultures grown at a salinity of 32, and the lack of a relationship between the growth rate and toxicity for the unialgal cultures grown at the lower salinities (10 and 20).

This purported allelopathic compound has been found in the filtrates of *H. akashiwo,* and was indentified as a possible tannin-like ectocrine (Pratt 1966). This was later expanded by Yamasaki *et al.* (2009), who described these filtrate compounds as allelopathic polysaccharide-protein complexes (APPCs) with a molecular size > 10^6 Da. Yamasaki *et al.* (2009) further proposed that these APPCs function as glycoproteins that bind to the cell surface of *Skeletonema costatum* for induction of their allelopathic effect. Since a measurable increase in ichthyotoxicity was found in our contaminated cultures of *H. akashiwo*, compared to the unialgal cultures, this ichthyotoxin may in fact be the same compound as the allelotoxin reported in these previous studies, but stimulated in response to different environmental factors. However, additional research is required to confirm this possibility.

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The effects of acidity and nutrient stress on the toxicity of *Heterosigma akashiwo*

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Abstract

Heterosigma akashiwo (Y. Hada) Y. Hada ex Y. Hara et M. Chihara is a bloom-forming raphidophyte that causes finfish kills in temperate coastal waters worldwide. Although the toxic mechanism has yet to be fully characterized, toxicity is not constitutive, but rather under environmental control (i.e. salinity). In this study we measured growth and toxicity of cultures of *H. akashiwo* isolate NWFSC-513 across a range of acidity (pH) and nutrient regimes. Four acidity levels were tested (pH = 8.2, 8.1, 7.8, and 7.4) with the addition of nitrogen ranging from 10 to 880 µM. In addition, we examined if toxicity varied as a function of N substrate (nitrate, ammonium, and urea) used for growth. Growth rates were determined by cell density over time, and toxicity quantified using the immortal cell line RTgill-W1 assay. This isolate of *H. akashiwo* was able to grow well with all three nitrogen substrates. Forcing the cells to grow at lower pH levels did not alleviate cell toxicity. Toxicity increased with nitrogen concentration, with the greatest increase occurring between 10 and 100 µM. Based upon these findings, acidification and eutrophication of coastal zones will only exacerbate the incidence and negative impact of *H. akashiwo* blooms.

Keywords: Heterosigma akashiwo, toxicity, pH, nutrients, ocean acidification, RTgill-W1 assay

Introduction

Harmful algal blooms (HABS) of *Heterosigma akashiwo* have caused mortality in both natural and cultivated populations of finfish in temperate coastal regions worldwide (Horner *et al.* 1997; Ajani *et al.* 2001). The number of fish killed by a single bloom event can be over 65 million, with an associated loss of revenue of 2-6 million USD (Rensel 1995). However, not all blooms of *H. akashiwo* demonstrate toxicity (de Boer *et al.* 2004). Although the variable expression of the toxic mechanism has yet to be fully characterized, it is often postulated to be a stress-response to some biogeochemical condition(s) (Twiner *et al.* 2001; Fu *et al.* 2010; Ling and Trick 2010; Fredrickson *et al*. 2011).

Changes in pH and nutrient regimes in coastal environments can result from natural processes, such as the upwelling of acidified, high nutrient waters (Feely *et al.* 2008) or anthropogenic influences (e.g., Davidson *et al.* 2012). Altered environmental conditions such as lower pH levels have been found to cause changes to the physiology and toxicity of other HAB species (e.g., Sun *et al.* 2011; Tatters *et al.* 2012; Van de Waal *et al.* 2014), and it is well known that nutrient limitation (e.g., Granéli and Flynn, 2006) is a common trigger for enhancement of toxicity in many HAB species, including *H. akashiwo* (Cochlan *et al*., 2014). However, absolute nutrient concentrations are not the only condition requiring attention. The form of the nutrient in question also plays a role in *H. akashiwo* growth/response. *H. akashiwo* have been found to exhibit preferential uptake of nitrogen as ammonium and urea compared to nitrate (Herndon and Cochlan 2007), so it is not unreasonable to suggest that toxicity could also vary as a function of the N substrate supporting cellular growth. This study documents the effects of pH and nitrogen concentrations/forms on the growth and toxicity of cultures of *H. akashiwo* isolate NWFSC-513.

Methods

The isolate *H. akashiwo* NWFSC-513, originally isolated from the Salish Sea – the estuarine system on the North American west coast consisting of the Strait of Juan de Fuca, Puget Sound, and the Strait of Georgia – was grown in unialgal batch cultures containing nutrient-enriched ESAW media (Harrison *et al*. 1980; Berges *et al*. 2001). Cultures were grown at 19°C under cool white fluorescent light, at a constant irradiance of 65-80 µmol photons m^{-2} s^{-1}. For experimental treatments, media was enriched with nitrate (as $NaNO_3$),

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ammonium (as NH_4Cl), or urea (as CH_4N_2O) as the source of nitrogen. Nitrogen concentrations were maintained at 10 µM N across all three nitrogen sources, in a constant 12:1 ratio of nitrogen to phosphorus. In a follow-up nutrient-limitation experiment, nitrate concentrations were 880, 400, 200, 125, 100, and 10 µM N. For all experimental treatments HEPES buffer was added to the media to a final concentration of 20 mM in order to maintain desired pH levels (Harrison and Berges 2005). Sodium hydroxide (2 M NaOH) and hydrochloric acid (4.1 M HCl) were titrated into the media to attain the experimental pH levels of 8.2, 8.1, 7.8 or 7.4. Stock cultures of *H. akashiwo* NWFSC-513 in stationary phase were used as a 10% (v/v) inoculum for all experiments. Each experiment was executed in triplicate, for a total of 30 samples per experiment. The experiment was also performed with non-concurrent triplicates.

Specific growth rates were measured by sampling every 12 hours ± 2 hours for cell density and determined using flow cytometry (BD Accuri™ C6 Flow Cytometer). Log transformed cell density values were plotted against time to establish the time of maximum rate of change of cells (the exponential growth phase). The slope of the line during the exponential phase was used to calculate the specific growth rate using the exponential growth equation (Guillard 1973).

The rainbow trout gill cell line (RTgill-W1) assay was used to determine the level of toxicity expressed by *H. akashiwo* cells. Procedures were followed as described by Dayeh *et al.* (2003) and Dorantes-Aranda *et al.* (2011), except as outlined below. Gill cells concentrations were adjusted to 1.50×10^5 cells mL^{-1} with the addition of L-15 complete media in a 96-well lidded plate and allowed to attain confluence. They were then exposed to 2×10^5 cells mL^{-1} of lysed algal cells. Lysed cells were used because *Heterosigma* toxicity *in vitro* has been shown to require both fractured cell wall and periplasmic components (Ling and Trick 2010; Powers *et al.* 2012). Differences in metabolic activity were detected following the addition of Presto Blue (Life Technologies Inc., Burlington, ON, Canada) on a fluorescence spectrophotometer plate reader (Agilent Technologies, Santa Clara, CA). Metabolic activity was used to represent gill cell viability and calculated using the following formula (Dayeh *et al*. 2003):

$$\text{GC Viability (\%)} = \left(\frac{\text{(Exp. RFU} - \text{Hydrogen Peroxide Control)}}{\text{Average (Positive Control} - \text{No Cell Control)}}\right)$$

Results were reported as gill cell toxicity using the following formula:

$$\text{GC Toxicity (\%)} = 100 - \text{\% Viability}$$

Results and Discussion

Growth rate variability as a function of pH and nitrogen form.

Specific growth rates of *H. akashiwo* NWFSC-513 were not impacted by pH, but varied according to the form of nitrogen used for growth when grown at 10 µM N, 11 µM $FeCl_3$ and 12:1 N:P, with nitrate yielding the highest specific growth rates across the range of pH treatments [Fig. 1.].

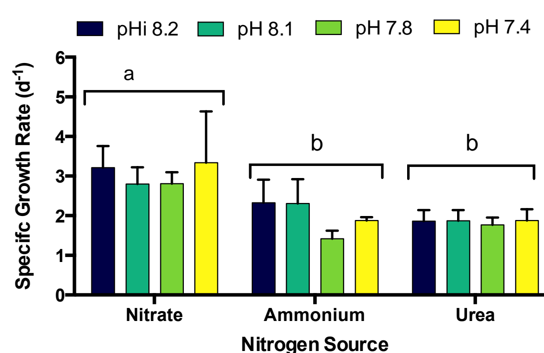

Fig. 1. Specific growth rates of *H. akashiwo* grown at various pH levels (8.2, 8.1, 7.8 and 7.4) and nitrogen substrates (nitrate, ammonium and urea) at 10 µM. pHi indicates an unbuffered culture where the pH changes over the experimental period. Error bars indicate one SD and letters represent statistically significant differences between treatments (N=6).

Specific growth rates with nitrate as the N-source were significantly higher than ammonium and urea [Fig. 1; $F(2,24) = 18.94$, $p < 0.0001$; post-hoc Tukey's test]. Rates with ammonium and urea did not differ significantly from each other. This finding contrasts previous work on a different isolate where nitrate yielded the lowest growth rates (Herdon and Cochlan 2007). This could point to physiological variabilty between isolates as shown by Fredrickson *et al*. (2011).

Growth of *H. akashiwo* NWFSC-513 did not appear to be negatively selected against under any of the N-sources or pH levels tested, as specific

growth rates were high and remained above 1.4 ± 0.2 d^{-1} in all treatments. Additionally, efficiency of use for the three N substrates tested, based on achieved cell yield, did not appear to be affected by the pH of the system. This result indicates that coastal waters experiencing fluctuations in pH and/or the form of available nitrogen are unlikely to limit the presence of *H. akashiwo*. The ability of the cells to maintain growth across a range of pH levels could also contribute to the unique vertical and horizontal migration exhibited by *H. akashiwo*, strains that have been shown to migrate through relatively strong pH gradients in some regions (Band-Schmidt *et al*. 2004; Feely *et al*. 2008). Furthermore, this apparent resilience to lower pH values may provide *H. akashiwo* a competitive advantage over co-occurring phytoplankton species demonstrating decreased growth rates as a result of drops in pH (Fu *et al*. 2008). Many studies seem to indicate that in general, coastal marine phytoplankton will be largely resilient to reduced pH to as low as 7.0 (Berge *et al.* 2010, Fu *et al*. 2010, Sun *et al*. 2011).

Toxicity as a function of the concentration of nitrogen and pH.

The ichthyotoxicity of *H. akashiwo*, grown on different nitrogen concentrations, was measured to evaluate how *H. akashiwo* may respond under possible future nutrient-rich and low pH oceanic regimes. Toxicity of *H. akashiwo* could greatly impact natural finfish populations as well as caged fish in aquaculture facilities. Nitrogen (as nitrate) concentrations tested were 10, 100, 125, 200, 400 µM; all concentrations lower than typically tested in laboratory cultures (e.g., 880 µM is the N concentration used in ESAW medium (Harrison *et al*. 1980; Berges *et al*. 2001)). The pH levels examined were 8.2 and 7.4, representing current average ocean surface and the lowered pH of surface waters in the Eastern boundary upwelling system (EBUS) projected for 2100, respectively (IPCC 2007; Feely *et al*. 2008).

A strong positive relationship between toxicity and nitrogen concentration was observed (Table 1.), which contrasts the observation of enhanced toxicity during nutrient-limited conditions observed for most HAB species, including *H. akashiwo* (e.g., Cochlan *et al*. 2014).

In terms of peak toxicity, there appears to be a rapid increase between 10 and 100 µM [Figure 2.] After this point, peak toxicity remains above 90% gill cell death for all other concentrations tested. At 10 µM N there was a wide range in toxicity, from 54.5 to 82.2% gill cell death, compared to a relatively narrow range of 87.7 to 97.7% gill cell death between 100 and 880 µM N [Figure 2.]. The range of pH tested did not significantly affect the toxicity of the *H. akashiwo* to the Rainbow Trout gill cells [Table 1; Figure 2.].

Table 1. Spearman's correlation coefficients for *H. akashiwo* toxicity to RT-gillW1 versus two different experimental variables tested (N concentration and pH) as well as measured specific growth rates. Significant correlations are marked in bold.

Variable	by Variable	Spearman's ρ	p-value
Toxicity	**µM N**	**0.7125**	**< .0001**
Toxicity	pH	0.0824	0.6432
Toxicity	growth rate	0.1812	0.3052

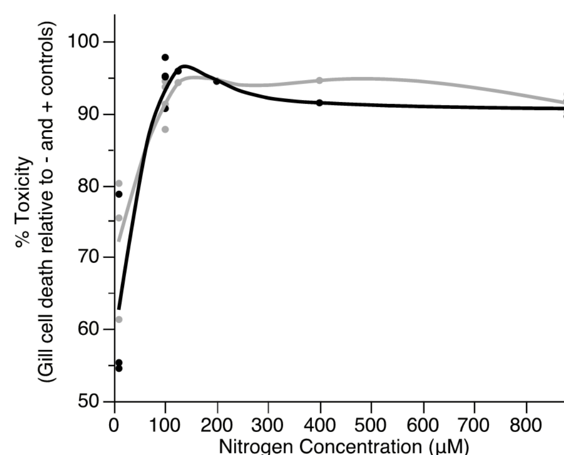

Fig. 2. *H. akashiwo* toxicity at pH 8.2 (black) and 7.4 (grey) across nitrogen concentrations of 10, 100, 125, 200, 400 and 880 µM. Toxicity values represent peak toxicity as measured using the RTgill-W1 assay. A cubic spline (λ = 0.05) has been applied over individual data points.

Although nitrate is typically found at concentrations < 40 µM in coastal zones, including upwelling systems (Codispodi 1983), in highly impacted areas and many estuarine systems the ambient concentrations of nitrate and ammonium are found in excess of 100-200 µM

(e.g., Nixon and Pilson 1983; Collos and Berges 2004). The results of the present study suggest such eutrophic regions could be prone to highly toxic blooms of *H. akashiwo*, and pH fluctuations would not dampen their level of ichthyotoxicity.

Conclusions

Based on the results of this laboratory culture study using *H. akashiwo* strain NWFSC-513, upwelling zones and terrestrial run-off could provide nutrient (namely nitrogen) concentrations to support toxic blooms of *H. akashiwo*. However, *H. akashiwo* growth seems to be largely resilient to changes in pH and nitrogen substrate, which will continue to fluctuate temporally and spatially in coastal regions worldwide.

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Characterizing toxic activity from *Heterosigma akashiwo*: a tale of two assays

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Abstract

Blooms of the raphidophyte, *Heterosigma akashiwo* (Y. Hada) Y. Hada ex Y. Hara et M. Chihara, have caused severe economic damage to fish farms in the inland waterways of Washington State, USA, and British Columbia, Canada, and are believed to be increasing in frequency and severity. In our study, two laboratory tests were used to characterize *H. akashiwo* toxicity - a modified rainbow trout gill cell assay and embryonic and larval zebrafish exposures. The gill assay demonstrated that the *H. akashiwo* toxin is primarily intracellular, highly soluble in methanol and ethyl acetate, and pH stable, with no loss of activity upon storage at -20°C. Stationary phase extracts from *H. akashiwo* culture were used to characterize the toxin's specific cellular targets on the development of zebrafish. At 48-hour postfertilization (hpf), intrinsic and specific effects to cardiomyocytes included reduced heart rate and atrial dilation, leading to pericardial edema. Zebrafish heart chambers formed normally, suggesting that the *H. akashiwo* toxin does not affect early cardiac development but is a physiological poison. In summary, the non-labile toxin from *H. akashiwo* is a largely intracellular, medium-to-low polarity organic compound that causes impairment of cardiac function in fish, possibly through impacts on cellular Ca^{2+} homeostasis.

Keywords: Heterosigma akashiwo, raphidophyte, toxicity, fish kill, zebrafish assay, gill cell assay

Introduction

Recurring threats from the raphidophyte, *Heterosigma akashiwo* have caused extensive devastation ($2-6 million USD per episode) to wild and net-penned fish of Puget Sound, Washington. The toxic activity of *H. akashiwo* has been attributed to the production of reactive oxygen species, brevetoxin-like compounds, excessive mucus, or hemolytic activity; however these mechanisms are not expressed consistently in all fish-killing events or cultured strains (e.g. Yang *et al.* 1995; Khan *et al.* 1997; Oda *et al.* 1997; Twiner and Trick, 2000). The difficulty of conducting research with active, toxin-producing field populations of *H. akashiwo* has resulted in conflicting findings from those obtained in lab culture studies, thereby limiting the ability of fish farmers to respond to these episodic blooms. However repeated studies have suggested that a neurotoxin is produced which causes hypoxic conditions of the blood and tissues as well as asphyxiation (e.g., Oda *et al.* 1997; Twiner and Trick 2000; Twiner 2002; Marshall *et al.* 2005; Khan *et al.* 1997). The goal of this study was to identify the primary neurotoxic element(s) associated with fish-killing *H. akashiwo* blooms, and thereby, to provide managers with the fundamental tools needed to monitor the toxicity associated with these harmful events.

Material and Methods

***Heterosigma* cell isolation and culture:** Culture methods followed those of Guillard (1995). Single *H. akashiwo* cells were isolated from net tow samples. Cultures were grown in filter-sterilized natural seawater, enriched with nutrients according to Berges *et al.* (2001, 2004), with modifications following Cochlan *et al.* (2008). Nitrate, the sole nitrogen source, was reduced from 550 to 300 μM and silicate was not added. Cells were grown to mid-exponential phase through multiple transfers before testing for toxicity using the gill cell assay. *H. akashiwo* isolate NWFSC-513 was harvested during early stationary phase when *H. akashiwo* toxicity was highest (Cochlan *et al.*, 2014) and used for solvent extraction experiments.

Solvent extraction of cells: Cells of *H. akashiwo* were pelleted by centrifugation at 1000 *g* for 10 min. Pellets were extracted in 4 mL methanol, the supernatants SpeedVac™ concentrated to dryness and reconstituted with distilled H_2O (dH_2O) to a final 1% methanol solution. This aqueous solution was partitioned using a separatory funnel through a series of immiscible solvents in the following order: hexane, ethyl acetate, dichloromethane (Fig. 1). Each extract (including the final dH_2O extract) was assayed using gill cell assay and compared to a control (no cell, solvent only) and the initial methanol extract.

Gill cell assay: Rainbow trout gill cells (RTgill-W1) from the American Type Culture Collection (CRL-2523) were exposed to NWFSC-513 extracts using the method of Dorantes-Aranda *et al.*, (2011) with the following modifications: NWFSC-513 was pelleted and extracted in 100% methanol, speed vacuumed to dryness and reconstituted in L15/ex medium, then applied on plated RTgill-W1 cells. Extracts resulting from liquid-liquid partitioning were processed similarly and resuspended in L15/ex prior to testing in gill cell assays.

Cell pumping system: A bloom of *H. akashiwo* at Cap Sante Marina Marina (48° 31' 5" N, 122° 36' 1"W) in Anacortes, northern Puget Sound, WA was collected in June 2014 using a polyaromatic adsorbent DIAION™ resin (HP-20, Mitsubishi Chemical Corp., Tokyo, Japan) in a large-scale pumping system described by Rundberget *et al.* (2007). Seawater was pumped continuously for 54h at a flow rate of 360 L h^{-1}. An average of 50 x 10^6 *H. akashiwo* cells L^{-1} were collected over the period of the bloom, resulting in ca. 1 x 10^{12} cells per g resin. During the pumping period, *H. akashiwo* was the dominant species, with *Prorocentrum lima* and small unidentified flagellates present at <100 cells L^{-1}.

Solvent extraction of resin: Exposed resin (2g) was rinsed with 30 mL H_2O at 1mLmin^{-1} to remove salts then eluted with 6 mL methanol at a rate of 0.5 mL min^{-1}. The extract was dried via speed vacuum and N_2 stream then diluted with 18 mL dH_2O to a final 1% methanol solution. The extract was partitioned with hexane and ethyl acetate as describe above (solvent extract of cells section), evaporated under a stream of N_2, resuspended in 1% methanol in zebrafish water

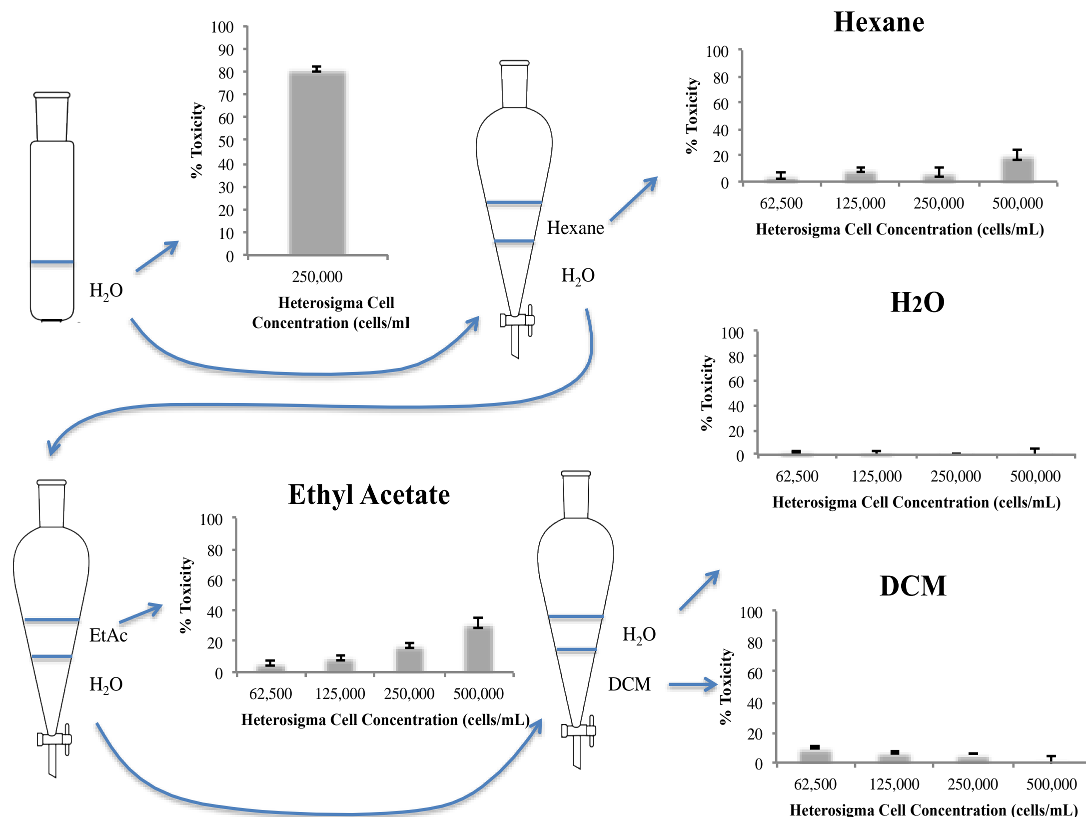

Fig. 1. Liquid-liquid partitioning of *H. akashiwo* strain NWFSC-513 harvested during stationary phase. Error bars on plots indicate ± 1 SD (n = 3) of replicate determinations.

(deionized and reverse osmosis filtered water amended with Instant Ocean Salt to a conductivity of 1500 μS cm^{-1}) for zebrafish exposure. Concentrations used for exposures (in triplicate) ranged from 4-15 x 10^6 *H. akashiwo* cell-equivalents per treatment.

Zebrafish exposures: Zebrafish embryos (n=15) at 256-cell stage (2.5 hpf) were placed in 5 ml zebrafish water in 6-well glass plates (Corning, Costar 3516) and kept in the dark at 28.5°C with water replacement every 24 h until 72 hpf. Controls were zebrafish water containing 1% methanol and zebrafish water alone. Digital video clips of embryos were collected from each treatment using a Nikon SMZ800 stereomicroscope or a Nikon Eclipse E600 and a Unibrain Fire-I800 camera with BTV Carbon Pro software as described in Incardona *et al.* (2014). For each treatment, both heart rate and percentage of zebrafish with edema were recorded (n=15).

Results and Discussion

The ethyl acetate extract of NWFSC-513 culture resulting from liquid-liquid partitioning showed the greatest dose-dependent toxicity in the gill cell assay compared to other fractions. About 25% of the activity observed in the original methanol extract was retained in the ethyl acetate fraction (Fig. 1). Typically, when extracted sequentially in this manner: the hexane fraction contains very lipophilic compounds (e.g. fatty acids); the ethyl acetate fraction contains the less polar uncharged molecules (e.g. pectenotoxins); the

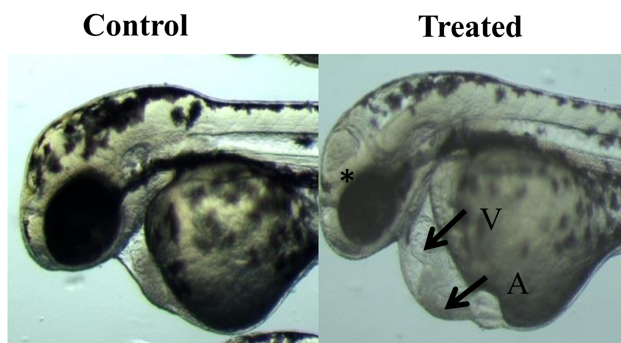

Figure. 2. Zebrafish exposed to *H. akashiwo* extract 2-48 hpf shows severe edema, hydrocephalus (*), and atrial (A) dilation. V=ventricle

dichloromethane fraction contains the more polar organic molecules (e.g. okadaic acid); and, salts and highly polar or ionic organic compounds (e.g. yessotoxins) remain in the water fraction. Resin extracts from the high density *Heterosigma* bloom (June 2014) also showed the highest activity in the ethyl acetate fraction and, in general, showed

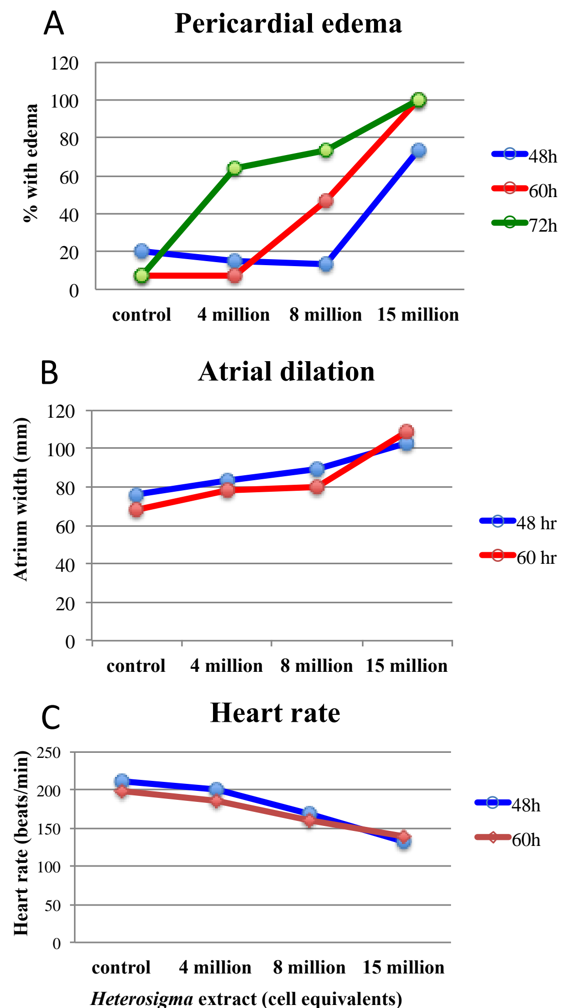

Fig. 3. Dose dependent cardiac effects of *H. akashiwo* ethyl acetate extracts on zebrafish pericardial edema (A), atrial dilation (B), and heart rate (C). Control heart rate is 211 beats per minute (bpm) and highest exposed is 132 bpm at 48 hpf. Average std. dev. in (A) = 2 bpm (n=15).

more reliable toxicity than cultures, in particular because the latter appeared to lose their toxicity after ~1 year. For this reason, resin extracts were used for zebrafish exposure experiments. Zebrafish embryos exposed to the HP20 resin ethyl acetate extract showed severe pericardial edema (Figs. 2, 3), atrial dilation, and hydrocephalus shown by a clear space in the 4th ventricle of the brain (Fig. 2). The incidence of edema and decrease in heart rate was dose-dependent with exposure to HP-20 resin extract (Fig. 3). Together, these data suggest that *H. akashiwo* produces a stable toxin that is

moderately lipophilic. It is proven to be a chemical entity because toxic activity is recovered after drying down in methanol and resuspended in assay buffer. Solubility in ethyl acetate suggests that it may be a relatively non-polar, neutral polyether-like compound. The toxin causes bradycardia, edema and atrial dilation has a negative chronotropic effect in zebrafish. Because the cardiac-specific effects occurred by 48h exposure and the heart chambers were formed normally, the effect is intrinsic to heart development and not a secondary effect, such as at the acetylcholine receptor which appears at a later stage in zebrafish development. This evidence suggests that the *H. akashiwo* toxin is a physiological poison.

Twiner (2002) proposed that *H. akashiwo* extracts had an effect on Ca^{2+} release by muscarinic-1 transfected sf9 insect cells pretreated with lanthanum, a Ca^{2+} channel blocker (Twiner 2002). The effects of *H. akashiwo* extracts on cardiac function that we observed also point to the possibility that *H. akashiwo* impairs Ca^{2+} homeostasis. The negative chronotropic effects shown by *H. akashiwo* extracts on zebrafish are consistent with Ca^{2+} blockage during the plateau phase of the action potential. However, this study is not a confirmation of *H. akashiwo* impacts solely on Ca^{2+} homeostasis as the zebrafish exposures resulted in mixed phenotypes, suggesting other toxic mechanisms. Head-first hatching which is characteristic of brevetoxin effects on zebrafish embryos (Kimm-Brinson & Ramsdell 2001), was not observed, suggesting that *H. akashiwo* toxins are not brevetoxins, as has been suggested (Khan *et al.* 1997).

Acknowledgements

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The sugar kelp *Saccharina latissima* is a potential source of the emerging toxin, Pinnatoxin-G, in cold waters

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Abstract

The presence of the cyclic imine toxin, pinnatoxin-G, was unambiguously identified in the sugar kelp *Saccharina latissima* from Norway by liquid chromatography coupled to mass spectrometry analysis, running under several operation modes. Enhanced product ion scans acquired at 30, 50, 70 and 90 eV of collision energies from the precursor $[M+H]^+$ of PnTX-G at *m/z* 694.5, matched with those obtained for the analysis of PnTX-G reference solution. Quantification was performed with the mass spectrometer operating in multiple reaction monitoring mode, with selected transitions *m/z* 694.5 > 676.4 and 694.5 > 164.1. Six-level calibration curves between 0.19-38 ng $\cdot mL^{-1}$ showed good intra-batch performance: linear adjustment (r^2) between 0.9961-0.9999 and slope shift between 0.5- 5.7% among subsequent calibration curves. The concentration of PnTX-G in *S. latissima* under these conditions was estimated of 5.1 ± 0.4 $\mu g \cdot kg^{-1}$. This finding strengthens the evidenced of a wide latitudinal distribution of pinnatoxins, and it suggests that kelp or seaweeds can be a potential ecological niche for benthic dinoflagellates producers of pinnatoxins in cold waters. Assessment on how food processing may affect to the levels of this toxin in manufactured food products containing kelp-based ingredients should be further investigated.

Keywords: marine toxins, cyclic imines, pinnatoxin, liquid chromatography-mass spectrometry, seaweed.

Introduction

Pinnatoxin-G (PnTX-G, Figure 1) belongs the group of marine toxins known as cyclic imines, a subclass of lipophilic marine toxins that can be accumulated in edible tissues of filter-feeding organisms (Otero *et al.* 2011). They are potent antagonist of nicotinic acetylcholine receptors, being the spirocyclic imine moiety responsible of the pharmacological activity (Duroure *et al.* 2011)

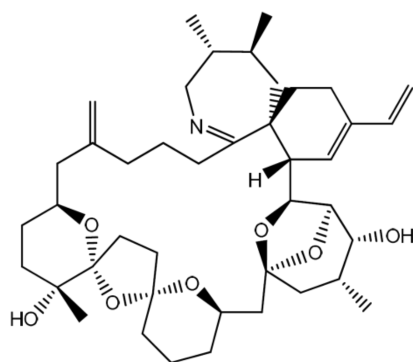

Fig. 1. Structure of Pinnatoxin-G.

To date, the only known producer of pinnatoxins is the benthic dinoflagelate *Vulcanodinium rugossum*, which has been only reported in warm or temperate waters of Australia, New Zealand and Japan (Rhodes *et al.* 2011), NW Mediterranean (Nézan and Chomérat 2011, Satta *et al.* 2013), South of China (Zeng *et al.* 2012), Mexican Pacific (Hernández-Becerril David *et al.* 2013) and Hawaii (Smith et al 2014). However, pinnatoxins have shown much wider latitudinal distribution and they have been found in shellfish and/or seawater of Norway (Rundberget *et al.* 2011) and Easter Canada (McCarron *et al.* 2012). Despite their toxicity observed in mice, the European Food Safety Authority could not draw the risk associated to shellfish consumption containing these toxins because the limited toxicological records and the lack of exposure data (EFSA, 2010).

Material and Methods

Pre-released reference material of concentration ~1.9 $\mu g \cdot mL^{-1}$ was obtained from the NRC (Halifax, NS, Canada). For liquid chromatography-mass spectrometry (LC-MS) analyses, hypergrade acetonitrile (ACN) was used for separation and gradient grade HPLC methanol (MeOH) was used for extraction and for washing the injection needle. Both were purchased from (Fisher Scientific, Loughborough, UK). Ammonium hydroxide solution eluent additive for LC-MS (≥25% in H_2O) was purchased from Fluka

brand of Sigma-Aldrich (Steinheim, Germany). Ultrapure water (resistivity>18 MΩ·cm) was obtained from a Milli-Q water purification system (Millipore Ltd., Billerica, MA, USA).

Seaweed samples of *Laminaria digitata* (kelp) and *Saccharina latissima* (sugar kelp) were sampled by Hortimare (http://www.hortimare.com) within the framework of the ECsafeSEAFOOD project on May 24th, 2013, near to a Norwegian salmon fish farm in a fjord in Jovika, at the north of Bergen (61º03'28.5"N, 4º55'29.6"E; Figure 2).

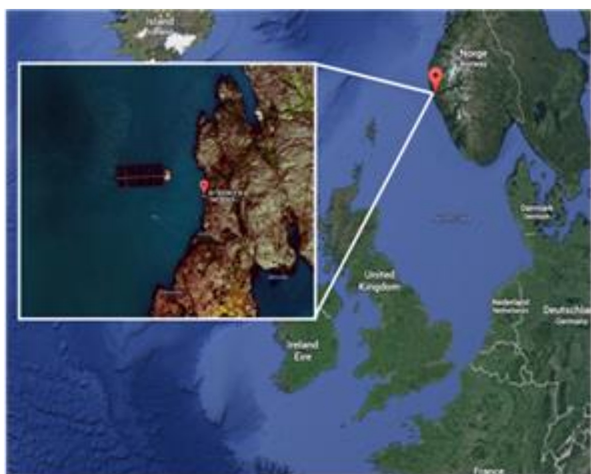

Fig. 2. Sampling location of the seaweeds at the north of Bergen, Norway.

Samples consisted on a pool of ca. 25 individuals, harvested at 5-6 and 0.5-1.0 meters depth for *S. latissima* and *L. digitata* species, respectively. The samples were first sun-dried after collection, and then freeze-dried at Hortimare's facilities in Netherlands. At IRTA in Spain, portions of 2.0 ±0.1 grams of lyophilized seaweeds were weight in triplicate, and extracted twice with 10 mL of MeOH using a high-speed homogenizer Ultra-Turrax T25 (IKA-Labortechnik, Germany). After centrifugation, the supernatants were reserved, combined and made up to a final volume of 20 mL with MeOH. A concentration step was carried out to enhance sensitivity. An aliquot of 10 mL was evaporated to dryness under N_2 stream in a TurboVap® (Zymark Corp., Hopkinton, MA, USA) and redissolved in 500 µL MeOH. The extracts were filtered through a 0.2- µm cut-off polyvinylidene fluoride (PVDF) membranes (Whatman Ltd., Maidstone, UK) previous to LC-MS/MS analysis. Initially, samples were analyzed with the LC-MS/MS method under alkaline elution conditions (Gerssen *et al.* 2009, García-Altares *et al.* 2013), method that is routinely applied for the analysis of shellfish samples from the surveillance program conducted in Catalonia. Separations were performed using an Agilent 1200 LC (Agilent Inc., Palo Alto, CA) at 30 ºC and a 500 µL·min^{-1} flow rate on a X-Bridge C8 column (2.1 x 50 mm, 3.5 µm) protected with a pre-column (2.1 x 10 mm, 3.5 µm) packed with the same stationary phase, both from Waters (Milford, MA, USA). A binary gradient elution was programmed with alkaline mobile phases (pH ~11). Channel A delivered H_2O and channel B delivered H_2O/ACN (10:90, v/v), both containing 6.7 mM of ammonium hydroxide. The gradient ran from 20 to 100% B over 8 min, hold it at 100% B for 1 min, came back to the initial conditions over 0.5 min and finally hold it at the starting conditions for equilibration before next injection, keeping a total run cycle of 12 min. Mobile phase eluting from column was diverted to waste for the first 1 min of analysis. To avoid carry-over between injections, a 100% MeOH was used as solvent for needle washing and runs of blank MeOH were performed between samples. Injection volume was 10 µL and the autosampler was set at 4 ºC. The 3200 QTRAP® mass spectrometer (AB Sciex, Concord, ON, Canada) was coupled to chromatographic eluent through an atmospheric pressure ionization-electrospray ion source TurboV® (AB/Sciex). Collision and source parameters were as follows: 20 psi curtain gas, 5500 V (ESI+), 500 ºC nebulizer gas temperature, 50 psi nebulizer and heater gases, level 4 (arbitrary units) for collision-activated dissociation gas, 85 V of declustering potential and 55 eV of collision energy (CE) and 2600 V continuous electron multiplier. PnTX-G was monitored with two Multiple Reaction Monitoring (MRM) transitions at 694.5 > 676.4 and 694.5 > 164.1. A nitrogen generator NM20Z (Peak Scientific, Renfrewshire, Scotland) supplied all operation gases. Acquisition and control instrument was powered by the software Analyst.

Results and Discussion

A signal was found in the sample of *S. latissima* corresponding to the two MRM transitions of PnTX-G. This first detection was followed by *ad hoc* LC-MS/MS experiments aimed at confirming the identity of PnTX-G in the sample. Enhance product ion (EPI) spectra were acquired in the range *m/z* 150-700, taking as a precursor ion the species $[M+H]^+$ of PnTX-G (*m/z* 694.5), for a standard solution of PnTX-G of 38 ng·mL^{-1} and for the sample of *S. latissima*.

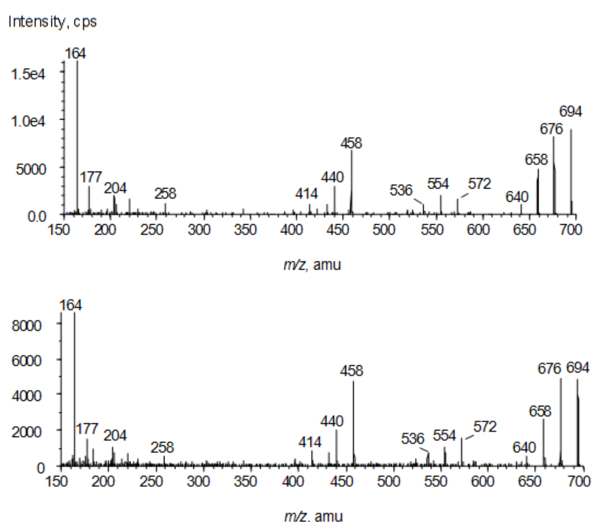

Fig. 3. Enhance product ion spectra from the precursor ion $[M+H]^+$ at *m/z* 694.5, acquired at 70eV CE of collision energy. Top: standard calibration solution with 38 ng·mL^{-1} of PnTX-G. Bottom: Sample of the kelp *S. latissima* containing PnTX-G.

Different EPI experiments were acquired at different CEs of 30, 50, 70 and 90 eV, in order to compare how the relative abundance in the spectra varied with CE, and whether these variations were consistent for both the reference solution and the PnTX-G in the sample. Thus, the presence of PnTX-G in *S. latissima* could be unambiguously identified, by comparison of the response for the standard of PnTX-G and the PnTX-G in the sample. Figure 3 shows an example of the EPI spectra at CE of 70 eV. Confirmation criteria fulfilled the requirements of the 2002/657/EC (EC, 2002) in terms of retention time (average of 6.6 min, < 2% variation) and in relative intensities of product ions in the full product ion spectra. Besides, the relative abundance among ions varied equally between the standard and PnTX-G in the sample when different collision energies were applied (data not shown). The fragment ions observed were in agreement with those proposed in the fragmentation pathways (Figure 4). Full scan in the range *m/z* 200-800 was acquired with the enhanced mass spectrometry (EMS) mode. Investigation on the presence of other pinnatoxin analogues was performed extracting the corresponding nominal mass of protonated ions $[M+H]^+$ for: PnTX-A (*m/z* 712.5), PnTX-B/-C (*m/z* 741.5), PnTX-D (*m/z* 782.5), PnTX-E (*m/z* 784.5), and PnTX-F (*m/z* 766.5) (Hess *et al.* 2013, Geiger *et al.* 2013, McNabb *et al.* 2012). However, none of these analogues could be identified in *S. latissima*. Additional experiments were performed with the precursor ion scan mode from the product ion *m/z* 164, common for all PnTX analogues, in the range m/z 650-850. This experiment could have revealed other potential unreported PnTX analogues. However, as a result only PnTX-G could be evidenced also in these experiments (data not shown). Quantitatively, a six-level calibration curve was performed with reference calibration solution of PnTX-G between 0.19-38 ng·mL^{-1}. The calibration curves showed good intra-batch performance: linear adjustment (r^2) between 0.9961-0.9999 and slope shift between 0.5- 5.7% among subsequent calibration curves. The limit of quantification was estimated ca. 0.2 µg·kg^{-1}; and the concentration of PnTX-G in *S. latissima* was estimated of 5.1 ± 0.4 or 5.3 ± 0.3 µg·kg^{-1}, taking MRM transitions for quantification *m/z* 694.5 > 676.4 or 694.5 > 164.1, respectively.

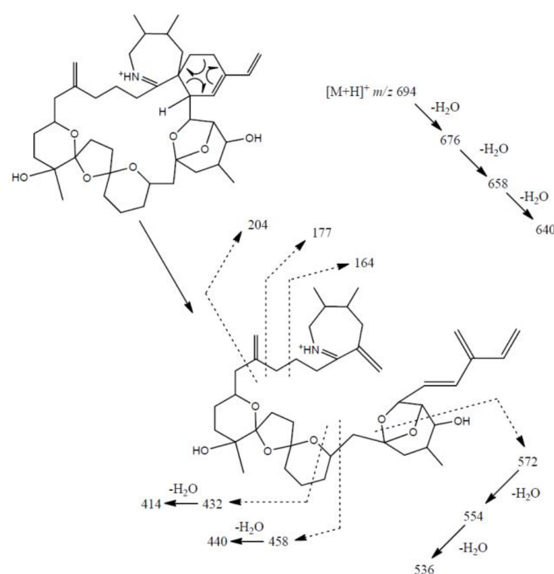

Fig. 4. Fragmentation pathways of Pinnatoxin-G.

The presence of PnTX-G in *S. latissima* seems to be more likely related to the presence of epiphytic benthic dinoflagellate living attached on the surface of this kelp, rather than postulate that *S. latissima* is the actual producer of PnTX-G. Unfortunately, the remote location of this sampling point from our laboratory meant it was not possible to get live fresh macroalgae to isolate the producer. As stated in the introduction section, *V. rugosum* is the only dinoflagellate know to produce pinnatoxins, which has been identified exclusively in warm or temperate waters. Our happenstance finding reinforces the evidences of a wide latitudinal distribution of PnTXs, and highlight the kelp or seaweed substrates as a potential ecological niche for benthic

dinoflagellates producers of PnTXs in cold waters. The impact that translocation of live fresh kelp with commercial interest may have on the spreading of benthic dinoflagellates among seas should be assessed, as this practice is gaining attention in seaweed farming (Peteiro *et al.* 2014). The sugar kelp *S. latissima* is considered an edible and tasty seaweed because of its sweet flavour and naturally occurring monosodium glutamate. It is popularly known as sugar kombu or kombu royale in Japan, where it has been traditionally used to enhance the flavour of broths and noodles (McHugh, D.J., 2003; Seaweed industry association, 2015). Processed, *S. latissima* is also used in food industry as thickening agent and in cosmetics industry. Bearing in mind the very low levels of few parts-per-billion of PnTX-G found in this work, a poisoning outbreak due to direct consumption of *S. latissima* is unlikely. Concentrations reported in shellfish usually ranged from ca. 10 to 100-fold higher than that observed in *S. latissima*, which could almost exclude this source of pinnatoxin-G as a concern from a food safety perspective. Besides, the consumption of raw kelp (non-processed) is expected to be much lower than shellfish consumption. However, it should be assessed how food processing could affect to the levels of pinnatoxin-G, as we cannot completely rule out the possibility that toxins could be transferred to consumers through non-conventional vector pathways, for instance by consumption of manufactured food products containing kelp-based ingredients.

Acknowledgements

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Pinnatoxin A affects both chick embryo spontaneous movements and skeletal bone development

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Abstract

Pinnatoxins belong to an emerging class of potent fast-acting marine toxins of the cyclic imine group, and are known to exert a selective potent antagonistic action on neuronal- and muscle-type nicotinic acetylcholine receptors. Studies on their biological effects have been favored by the development of robust synthetic approaches. In the present work using synthetic pinnatoxin A (PnTx A), a detailed study has been performed on the effects of this toxin on chick embryo development. In this report, we demonstrate for the first time that the administration of PnTx A at early embryo stages caused a reduction in both embryo size and bone density, as compared to embryos exposed only to the solvent used to dilute the toxin. Furthermore, PnTx A also affected spontaneous movements in chick embryos *in ovo,* as revealed by recording the intervals of time between movements. Further studies will be needed to unravel the mechanism(s) whereby PnTx A affects spontaneous movements, and thereby the maturation of the embryo skeletal system, but it is likely that such actions are related to the ability of the toxin to block selectively nicotinic acetylcholine receptors from the central and peripheral nervous system of the chick embryo.

Keywords: Pinnatoxin A; Toxicity; Chick embryo; Nicotinic acetylcholine receptors;

Introduction

At present, eight structurally related pinnatoxins (A-H) have been reported. These phycotoxins belong to a heterogeneous group of macrocyclic marine compounds, globally distributed, called cyclic imine toxins (for recent reviews see Molgó *et al.* 2014; Stivala *et al.*2015).

The peridinoid dinoflagellate *Vulcanodinium rugosum* has unequivocally been shown to be the producer of different pinnatoxins in distinct world locations (Nézan and Chromérat, 2011; Rhodes et al. 2011).

All pinnatoxins share the same common scaffold with a dimethyl substituted 7-membered cyclic imine as part of a spiroimine ring system, a 6,5,6-spiroketal ring system and a bridged ketal which is unique within the cyclic imine phycotoxins family. Successful strategies for the synthesis of these molecules, have been developed (Stivala and Zakarian, 2008; Nakamura *et al.* 2008; Aráoz *et al.* 2011).

Pinnatoxins are potent and selective inhibitors of both neuronal- and muscle-type nicotinic acetylcholine receptors (nAChRs), as revealed by electrophysiological and competition-binding studies (Aráoz *et al.* 2011; Hellyer *et al.* 2013), and are highly toxic in mouse bioassays when administered by intraperitoneal injection or by oral gavage (Munday *et al.* 2012).

The present study was designed in an attempt to determine whether pinnatoxin A (PnTx A) had teratogenic effects on the chick embryo, or exerted other toxic actions when administered at early stages of chick embryo development.

Material and Methods

PnTx A was synthesized as previously reported (Aráoz *et al.* 2011). Fertilized chick eggs of the JA 57 strain were incubated at 38 °C. After 1.5 days of incubation (embryonic day (E), E1.5), corresponding to the neural plate stage, when the formation of the central and peripheral nervous system takes place, a single microinjection of PnTx A was performed. At this stage, the chick embryonic development is similar to the Carnegie Stage 10, which corresponds to 28 days in the human embryo development.

To access the embryo, an opening was performed in the shell, which after microinjection was sealed with plastic tape to keep the normal embryo developmental conditions

The toxin was delivered, under visual control with a microscope, either as a single bolus of 100 µL (1-2 pmol) on the surface of the vitelline membrane at E1.5, or with a micro-pipette (Fig. 1) in a volume of 0.1 to 0.2 µL on the lumen of the neural tube at E4.5. Using the same approach, control embryos were microinjected with phosphate buffer saline solution containing the solvent (ethanol 0.025 v/v) used to dilute the toxin.

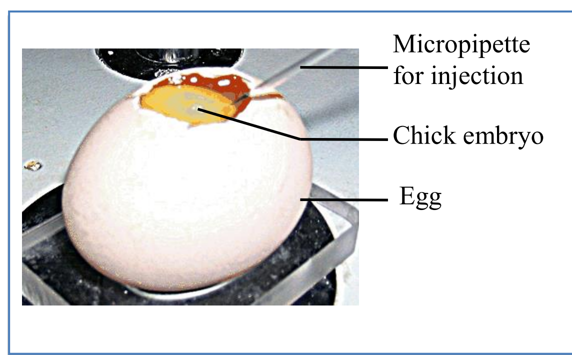

Fig. 1. Experimental model for testing the action of PnTx A on chick embryo development.

Skeletal development was evaluated after whole-mount Alcian blue staining. E8 embryos were fixed for several days at room temperature in ethanol 80% / acetic acid 20% / Alcian Blue 8X 0.015%. After extensive washing in ethanol 100%, cartilages were cleared with glycerol 20% / KOH 1% before macroscopic observation of embryo skeleton and cartilages.

In order to detect the spontaneous motor activity, E5 chick embryos were imaged *in ovo* with a camera, either 0.5 or 3 days after toxin or solvent administration. It should be noted that temperature was maintained constant during time-lapse recordings, to avoid thermal shocks that produce kinetic changes in the spontaneous motor activity.

Results and Discussion

As shown in Fig. 2, E8 embryos injected with PnTx A (at E1.5) were significantly smaller, and their bone density was drastically reduced, as compared to embryos exposed to the solvent used to dilute the toxin. Interestingly, no apparent development delay was observed in the treated embryos. Notably, digits and vertebrae were normally differentiated at E8 (Fig. 2), and no teratogenic effects were detected.

To assess if the defects in skeletal maturation could be linked to alterations in the spontaneous motor activity of embryos, the motility of E5 embryos was analyzed, in particular flexion-extension longitudinal movements (Fig. 3A) and transversal contractions (Fig. 3B).

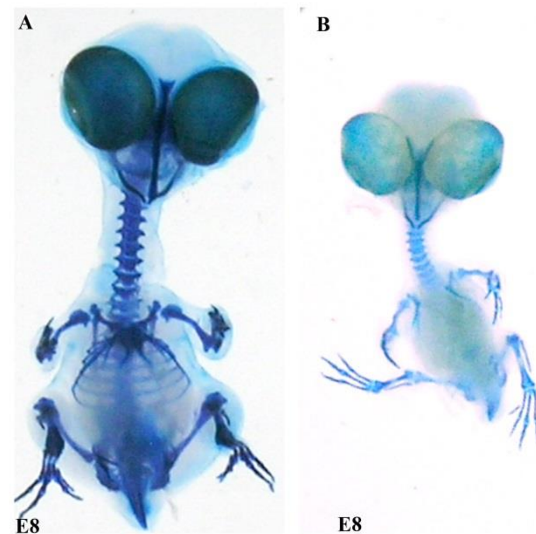

Fig. 2. Whole-mount skeletal preparations at E8 of control (A) and PnTx A-injected (B) embryos, showing the reduction of cartilage density after the action PnTx A.

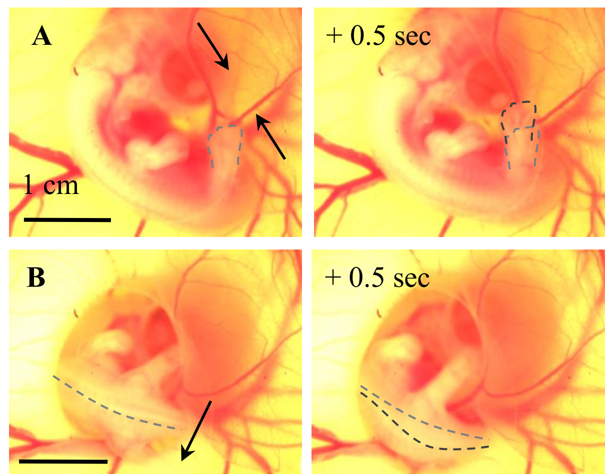

Fig. 3. Recordings of spontaneous movements in a E5 chick embryo. (A) Flexion-extension longitudinal and transversal movements (B), that are observed rhythmically, interspaced with latency periods of embryo inactivity.

Control E5 chick embryos exhibited spontaneous flexion-extension longitudinal movements, in conjunction with transversal contractions, similar

to those observed in 9-week old human fetuses. These spontaneous movements are considered to be of neurogenic origin (Hamburger, 1970). Movements recorded in E5 chick embryos were typically separated by latency periods varying from 20 to 30 seconds. However, 12 hours after PnTx A microinjection to E4.5 chick embryos, motility was greatly decreased..

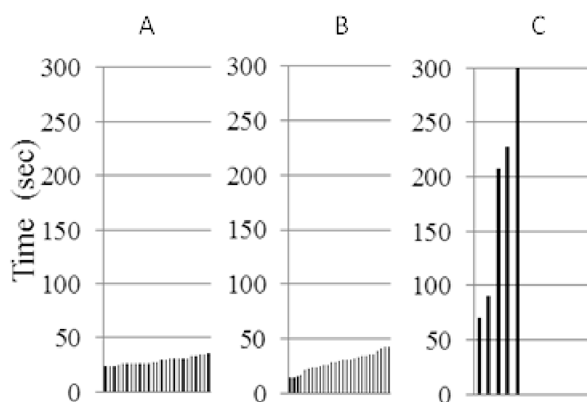

Fig. 4. Typical latency recording periods (in seconds) between spontaneous movements in 3 individual E5 embryos, under control conditions (A), after microinjection of the solvent used to solubilize the toxin (B) and during the action of PnTx A (C).

In some cases, embryos showed a complete loss of spontaneous motor activity To evaluate the frequency of spontaneous motility in E5 embryos, the length time of latency periods, between movements, was quantified in different embryos (n = 8) imaged for three periods of 5 minutes each. A typical example of the distribution of latency periods between spontaneous movements is shown in Fig.4.

Preliminary results indicated that both control embryos and embryos injected with the solvent used to dilute the toxin exhibited regular latency periods over the time of observation. In contrast, embryos injected with PnTx A, 12 hours before observation, had extensive latency periods, and some showed no movement at all despite being alive, as detected by their apparent heart beating. Our results are similar to those previously reported with α-bungarotoxin, a competitive antagonist of nAChRs (Oppenheim *et al.*, 2000), in which 12 hours after toxin injection to E5 embryos, the motility of embryos was greatly reduced. Different syndromes, like congenital myotonic dystrophy, or spinal muscle atrophy, in which fetal movements are impaired have demonstrated the crucial role of muscle contractions for normal bone and joint formation. Present results clearly show that PnTx A can act very early during chick embryo development (as soon as E1.5) by impairing spontaneous motor activity and thus leading to skeletal maturation defects.

Mechanisms whereby PnTx A impinges on embryonic development and motility require further characterization. In particular, we know that PnTx A binds with high affinity to both muscle-type ($\alpha 1_2\beta\gamma\delta$) and neuronal (α7, α3β2 and α4β2) nAChRs, being more selective for α7 nAChR subtype (Aráoz *et al.* 2011). Therefore, it would be of interest to determine the spatio-temporal expression pattern of some of these subtypes of nAChRs in the chick embryo at different stages of development.

The results presented reveal a detrimental effect of PnTx A on embryonic development, and our pilot study raises the question of a potential risk for fetuses that could be exposed to this environmental toxin.

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Harmful algal blooms affect early-life stages of Japanese pearl oyster, *Pinctada fucata martensii*

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Abstract

Pearl oysters, *Pinctada fucata martensii*, are commercially important bivalve molluscs in Japan. Their spawning and larval development seasons are coincident with harmful algal blooms (HAB), both toxic and noxious. The effects of experimental exposures to *Heterocapsa circularisquama*, *Alexandrium affine*, *Alexandrium catenella*, *Karenia mikimotoi*, *Karenia papilionacea*, *Heterosigma akashiwo*, *Chattonella marina* and *Chattonella antiqua*, on early embryo development and larvae of pearl oysters were studied. The dinoflagellates *Alexandrium affine*, *A. catenella*, and *H. circularisquama* affected embryo development. Complete inhibition of early embryo division was induced by non-PST producer *A. affine* and PST-producer *A. catenella* (saxitoxin and gonyautoxin-3). D-larvae were affected only by exposure to *H. circularisquama*. The results of the present study show that *H. circularisquama* is not the only species of HAB that affect pearl oyster early life history. Further studies are required to assess the effects of *A. affine*, *A. catenella* and other recurrent HAB in the cultivation areas of *P. fucata martensii*.

Keywords: embryo, larvae, pearl oysters, HAB, dinoflagellates, raphidophytes

Introduction

Harmful algal blooms (HAB) affect aquatic organisms via physical contact, production of toxins, and/or increased biomass (Landsberg 2002). Mass mortalities to sub-lethal effects have been well-documented in several aquatic organisms, including mammals, seabirds, fish, and shellfish, during HAB events. The impacts of HAB on early-life stages of commercially and ecologically important bivalve molluscs are largely unknown. Most of the studies considered the effects of harmful dinoflagellates on Trochophores and D-larvae of commercially important species of Pacific and eastern oysters, *Crassostrea gigas* and *Crassostrea virginica*, hard clams, *Mercenaria mercenaria*, and king and bay scallops, *Pecten maximus* and *Argopecten irradians* (Fig. 1).

In Japan, farms of the pearl oyster, *Pinctada fucata martensii*, are located in areas prone to recurrent HAB development and associated fisheries damage induced by several species of dinoflagellates and raphidophytes (Fig. 2). The toxic dinoflagellate *Heterocapsa circularisquama* is considered the highest threat to pearl oyster industry due to its potent toxicity to juvenile and adult pearl oysters (Matsuyama 2012), and its extensive cytotoxicity at low cell density to the early-life development of Japanese pearl oysters have already been shown (Basti et al. 2011, 2013). However, the effects of other HAB species that form blooms during the reproductive season of pearl oyster were not studied.

In the present paper, the effects of *Alexandrium* spp. *Karenia* spp., *Heterosigma akashiwo*, and *Chattonella* spp. on early-embryos and larvae of Japanese pearl oyster, *Pinctada fucata martensii*, were investigated. Additionally, *H. circularisquama* was also tested and used as a comparative reference.

Material and Methods

The exposure experiments were conducted at the facilities of K. Mikimoto and Co. LtD., Mie Prefecture, Japan. Eggs and spermatozoa were stripped from the gonads of sexually mature male and female pearl oysters, and fertilization was artificially induced (Basti et al. 2011, Basti et al. 2013). Freshly fertilized eggs were exposed to *Alexandrium affine* and *A. catenella* (5×10^2 cells/mL), *Heterocapsa circularisquama* (10^3 cells/mL), *Karenia mikimotoi* and *K. papilionacea*

A. Lincoln MacKenzie [Ed]. *Marine and Freshwater Harmful Algae 2014*. Proceedings of the 16th International Conference on Harmful. Algae. Cawthron Institute, Nelson, New Zealand and the International Society for the Study of Harmful Algae (ISSHA)

(10^4 cells/mL), *Heterosigma akashiwo* (5×10^4 cells/mL), and *Chattonella marina* and *C. antiqua* (10^3 cells/mL), in 6-well plates (50 eggs/mL, 5 mL/well), in duplicate.

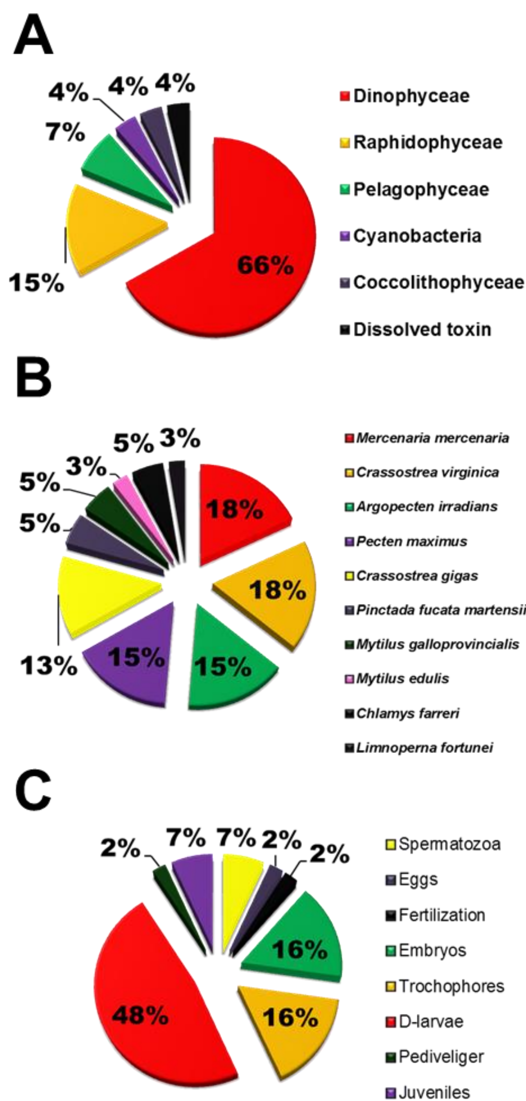

Fig. 1. Literature review of the effects of harmful algae on the early-life development of bivalves (1974–2013). (A) species of harmful algae, (B) species of bivalves, (C) developmental stages.

The harmful algae were cultured in F/2 or modified SW3 media, at 23°C, under a 12hL:12hD photocycle, and then their cells were harvested at the early to mid-stationary phases and added to the experimental seawater, at the desired cell densities.

Molecular confirmation of the strains of *A. affine*, *A. catenella*, *K. mikimotoi*, and *K. papilionacea* were conducted according to Nagai (2011) and Nagai et al. (2008, 2012). The profiles of paralytic shellfish toxins of *A. affine* and *A. catenella* were analyzed according to Ravn et al. (1995) and Oshima (1995).

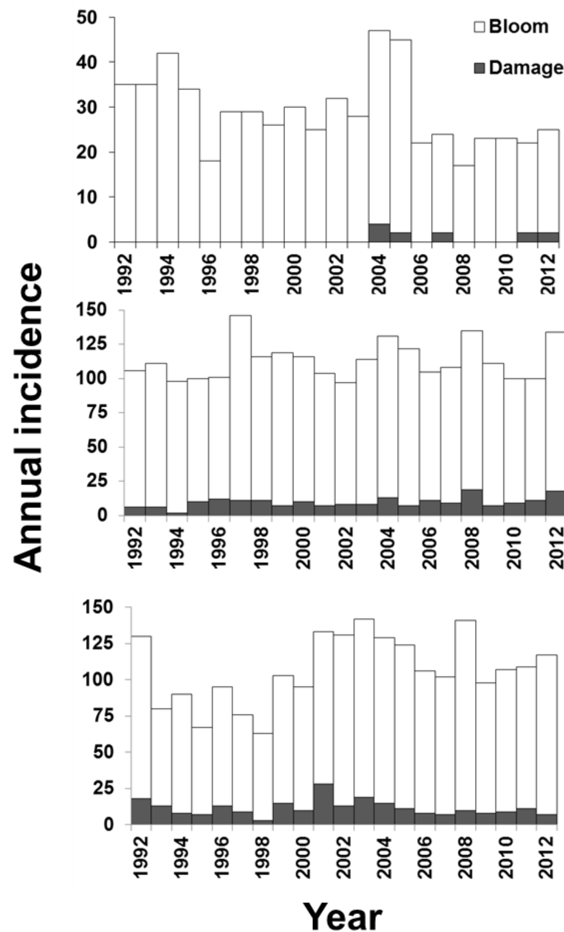

Fig.2. History of harmful algal blooms and associated fisheries damage in the cultivation areas of Japanese pearl oysters, *Pinctada fucata martensii*. (A) Mie Prefecture, (B) Seto inland Sea, (C) Kyushu Island.

The experiments were conducted at 23°C, in filtered and UV-treated seawater. The development of 4-cell embryos from fertilized eggs exposed to each harmful alga was assessed following 60 min of exposure.

In another set of experiments, D-larvae (24 h old) were exposed to the same harmful algae, at the same densities and under the same experimental conditions, to assess the effects on their swimming activity and mortality, within 24 h. In addition, 16 days-old eye-spot larvae were exposed to *H. circularisquama* at several densities to assess the effects on the activity and mortality following 24, 48, and 72 h of exposure to 10, 10^2, 5×10^2, and 10^3 cells/mL (Fig. 3).

The activity of both D-larvae and eye-spot larvae was assessed as the percentage of swimming larvae 5 min following gentle agitation of the well plates and resuspension of the larvae. Larvae were considered dead when there was complete absence of velar activity for 20s. All results were statistically compared (ANOVA and Student's *T*-test).

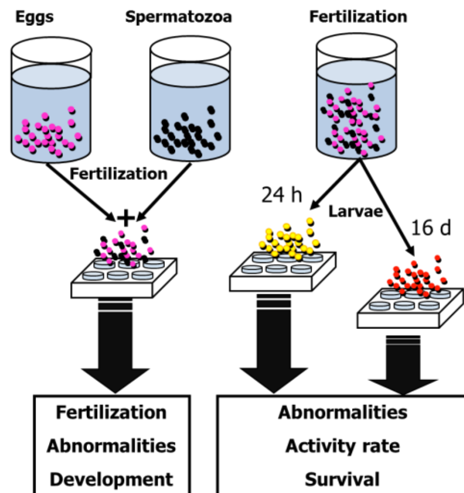

Fig.3. Schematic representation of the experimental protocol.

Results and Discussion

Exposure of freshly fertilized eggs to *Heterocapsa circularisquama*, *Alexandrium affine*, and *A. catenella* resulted in significant decrease in the percentage of 4-cell embryos following 60 min. In particular, exposures to *A. affine* and *A. catenella* resulted in a complete inhibition of embryo division and all fertilized eggs failed to reach the 4-cell embryo stage. Other harmful dinoflagellates and raphidophytes had no significant effects on early cleavage of Japanese pearl oysters (Table 1).

The activity of D-larvae was significantly decreased, and the mortality was significantly increased, only following 24 h of exposure to *H. circularisquama* (Table 2). Other harmful dinoflagellates and raphidophytes did not affect the activity and survival of D-larvae.The activity of eye-spot larvae (16 days-old) was significantly decreased following 24–72 h of exposure to *H. circularisquama* at 10–10^3 cells/mL (Fig. 4).

Table 1. Percentage of 4-cell embryos developed from fertilized eggs of *Pinctada fucata martensii* exposed to harmful algae.

HAB species	Density (cells/mL)	4-cell embryos
Heterocapsa circularisquama	10^3	74.5±1.9 *
Alexandrium affine	5×10^2	0.0±0.0 *
Alexandrium catenella	5×10^2	0.0±0.0 *
Karenia mikimotoi	10^4	39.3±7.5
Karenia papilionacea	10^4	43.5±6.5
Heterosigma akashiwo	5×10^4	69.7±7.2
Chattonella marina	10^3	55.0±7.1
Chattonella antiqua	10^3	90.0±10.0

(*) marks significance difference from respective control (P <0.05).

Table 2. Effects of harmful algae on activity (%) and survival (%) of D-larvae of *Pinctada fucata martenssi*.

HAB species	Density (cells/mL)	Activity	Mortality
Heterocapsa circularisquama	10^3	38.2±2.1*	15.2±1.5*
Alexandrium affine	5×10^2	97.5±1.0	0.1±0.0
Alexandrium catenella	5×10^2	94.9±0.2	0.0±0.0
Karenia mikimotoi	10^4	88.1±3.8	0.9±0.4
Karenia papilionacea	10^4	95.9±0.3	0.6±0.1
Heterosigma akashiwo	5×10^4	87.0±1.2	1.6±0.7
Chattonella marina	10^3	78.8±3.8	6.1±1.6
Chattonella antiqua	10^3	98.6±0.2	0.3±0.0

(*) marks significance difference from respective control (P <0.05).

Although mortalities were not observed, 45% of the eye-spot larvae exposed to 10^3 cells/mL of *H. circularisquama* for 72 h showed extensively lysed velum and hypersecretion of mucus when in contact with *H. circularisquama* cells (Fig. 5), as observed in a previous study with D-larvae of Japanese pearl oysters (Basti et al. 2011).

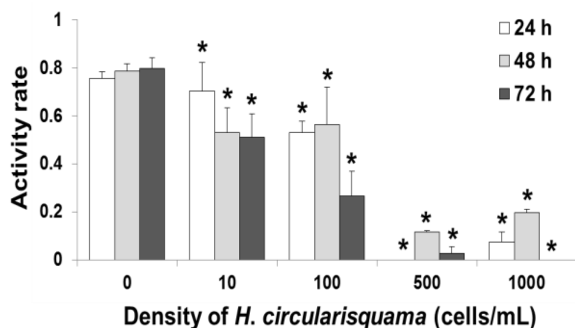

Fig. 4. Activity rate of eye-spot larvae exposed to toxic dinoflagellate *Heterocapsa circularisquama*. For (*) marks significance difference from respective control (P <0.05).

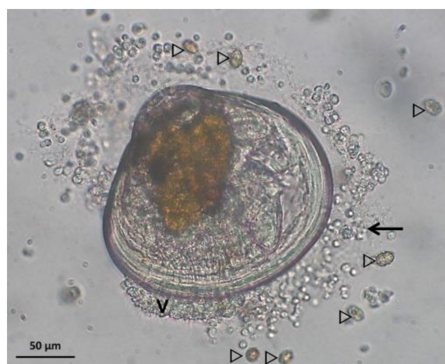

Fig. 5. Lysed velum (black arrow) of eye-spot larvae of Japanese pearl oyster, *Pinctada fucata martensii*, exposed to *Heterocapsa circularisquama* (72 h, 10^3 cells/mL). V: velum, arrow heads: vegetative cells of *H. circularisquama*.

The cosmopolitan harmful raphidophytes *H. akashiwo*, *C. marina*, and *C. antiqua*, and dinoflagellate *K. mikimotoi* are known cosmopolitan HAB associated with recurrent fish kills. Their exact mechanisms of toxicity are still not well understood. Although several toxins and metabolites were shown to affect fish, and in the case of *K. mikimotoi* shellfish, these had no effects on early embryos and larvae of Japanese pearl oysters, at the densities and exposure durations considered.

The most severe effects were observed for exposures to *Alexandrium* spp. and *H. circularisquama*. The strain of *A. affine* used in the present study was negative for PST, and *A. catenella* produced both saxitoxin (STX) and gonyautoxin-3 (GTX3); results showing the negative effects on embryo is not related to the production of paralytic shellfish toxins. In previous studies, detrimental effects on early-life stages of bivalve molluscs were found for a few species of *Alexandrium* that were also not necessarily associated with known toxins and other compounds (Matsuyama et al. 2001; Yan et al. 2001, 2003). In conclusion, the results of this study showed that, although *H. circularisquama* is the most potent HAB species tested to date for the early-life development of Japanese pearl oysters, *A. affine* and *A. catenella* severely affect embryos. Further detailed studies are necessary to address the effects of HAB on the reproduction and early-life development of Japanese pearl oyster.

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Discovery of enzymes related to marine polyether biosynthesis in dinoflagellate extracts

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Abstract

Ladder-frame polyethers have been assumed to be biosynthesized via polyene and polyepoxide precursors. However, the fused ether ring formation of marine ladder-frame polyethers has not yet been elucidated because of a lack of genetic information of dinoflagellates and the complexity of these structures. Brevisamide, which consists of a simple 6-membered ether ring, a dienal side chain and an acetylated amine, is produced by the toxic dinoflagellate, *Karenia brevis*. In order to search for an enzyme catalysing ether ring formation several proposed biosynthetic precursors of brevisamide, which have a linear olefin or an epoxide structure, were synthesized and incubated with extracts from the dinoflagellates, *Protoceratium reticulatum* and *Karenia* spp. Although desired 6-membered ether ring products were not detected in the extracts, some oxidation reaction products of terminal side chains were observed when the precursors were incubated with *Karenia* spp. extracts.

Keywords: cyclic ether, biosynthesis, enzyme, epoxide-opening, side-chain oxidation

Introduction

Marine ladder-frame polyethers produced by marine dinoflagellates are causative toxins of seafood poisoning and red tide incidents. They have unique structures and potent bioactivities. The biosynthesis of ladder-frame polyethers has been assumed as follows. A *trans*-polyene precursor is polyepoxidized and then resultant epoxides are cyclized in a cascade of an *endo-tet* epoxide-opening process by enzymes (Nakanishi 1985; Cho and Shimizu 1987). However, this mechanism still remains unproved because of a lack of genetic information of dinoflagellates and the complexity of large polyether structures.

In this study, biosynthetic enzymes such as epoxidase and an epoxide-opening enzyme were searched for using synthesized epoxide and olefine precursor compounds as substrates for the enzymatic reaction. A monocyclic ether brevisamide (**1**), which has a dienal side chain and an acetylated amine in the molecule, was isolated from the dinoflagellate *Karenia brevis* (Satake *et al*. 2008). The proposed biosynthesis of brevisamide is shown in Figure 1. In order to detect the enzymatic activity and prove the pathway, plausible biosynthetic precursors **2** and **3** of brevisamide were synthesized via Suzuki-Miyaura cross coupling and Katsuki-Sharpless asymmetric epoxidation (Shirai *et al.* 2010, 2014) and then incubated with dinoflagellate extracts.

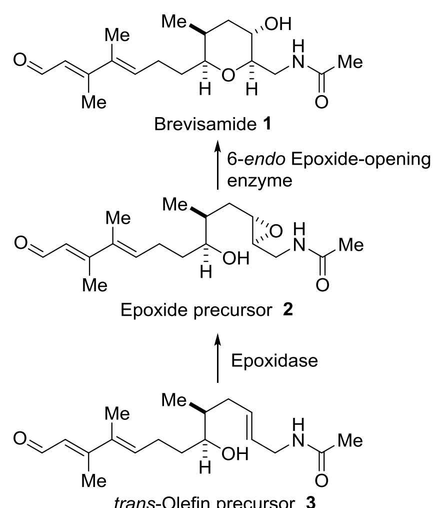

Fig. 1. Proposed biosynthetic pathway of brevisamide (**1**).

Material and Methods

Synthesis of epoxide and olefin precursors

The synthesis of epoxides (**2**, **6**, **7**, **8**) and olefin (**3**, **9**, **10**, **11**, **12**) precursors were reported in references (Shirai et al 2010, 2014).

A. Lincoln MacKenzie [Ed]. *Marine and Freshwater Harmful Algae 2014*. Proceedings of the 16th International Conference on Harmful. Algae. Cawthron Institute, Nelson, New Zealand and the International Society for the Study of Harmful Algae (ISSHA)

Preparation of cells of dinoflagellates.
Non-axenic unialgal cultures of *Protoceratium reticulatum* were carried out in 3 L Fernbach flasks in a seawater medium with GSe nutrients, which is a modified GPM medium containing 10.0 μM H_2SeO_3, at 17 °C. The culture solution of *P. reticulatum* was centrifuged (1500 g, 4 min, 4°C). Next the cell pellet was resuspended with washing buffer (50 mM HEPES (pH 7.4), 100 mM NaCl) and centrifuged (1500 g, 4 min, 4 °C). This operation was conducted two times. Cultures of the dinoflagellates *K. brevis*, *K. mikimotoi*, and *K. brevisulcata* were prepared and freeze-dried at the Cawthron Institute in New Zealand and sent to The University of Tokyo for enzyme extraction.

Incubation experiments.
Incubation buffer (50 mM HEPES (pH 7.4), 100 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM DTT, 1 mM PMSF, 2 mM EDTA) was added to the cells prepared above and then sonicated with a TAITEC ULTRASONIC PROCESSER VP-30 to afford a lysed extract. To the extract, a synthesised substrate was added and incubated at a constant temperature (27 °C) uisng a Yamato Shaking Bath BW100. After several hours the reaction mixture was quenched by adding methanol and centrifuged (19000 xg, 10 min, 4 °C) to pellet insoluble cellular debris.

LC/ESI-MS analysis.
The resultant incubated supernatants were analyzed by a JEOL JMS-T100LP“AccuTOF LC-plus” (LC/ESI-TOF-MS, ESI positive mode) liquid chromatography mass spectrometer equipped with either a Kanto Chemical mightysil RP-18-GP 50-1 or YMC-Triart C18 30 x 2.0 mmID column.

Results and Discussion

1-1 Search for an epoxide-opening enzyme
The plausible epoxide precursor **2** synthesized from propane-1,3-diol was incubated with extracts of the dinoflagellate *Protoceratium reticulatum*. This dinoflagellate was used because of its availability in the University of Tokyo and the fact that it produces the polycyclic ether yessotoxin,. Then the resultant products were analyzed by LC ESI-MS. A five-membered ring compound isobrevisamide (**4**) and an unknown compound were observed, but the desired product **1** was not detected (Figure 1 & 2). Compound **4** was generated by spontaneous 5-*exo* cyclization of **2**. The unknown compound was identified as a carboxylic acid derivative of isobrevisamide (**5**). Therefore, the existence of an aldehyde oxidase was indicated but an epoxide-opening enzyme could not be detected. This was probably due to the quick spontaneous 5-*exo* cyclization of the epoxide compound and substrate specificity.

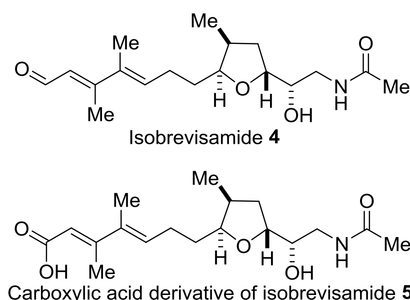

Fig. 2. Unnatural 5-*exo*-cyclization products from brevisamide precursors.

In addition to **2** and **3**, three epoxide precursor analogs **6**, **7**, and **8** (Figure 3) were synthesized and incubated with the cell preparations (*P. reticulatum*, *K. brevis*, *K. mikimotoi*, and *K. brevisulcata*). As with **2**, they also spontaneously cyclized to five-membered ring compounds in protic solvents, but were found to be stable in benzene. This demonstrated that a non-enzymatic 5-*exo* cyclization occurred, instead of a 6-*endo* cyclization. In addition, the slow oxidation at the aldehyde of **2** was also observed with the incubation with *K. brevis* and *K. mikimotoi*. And a trace amount of **4** was detected after incubating **6** with *K. brevisulcata*. This result showed that the dehydrogenation of the hydroxyl group occurred with *Karenia* spp.

1-2 Search for epoxidase
The olefin precursor **3** and its analogs **9**, **10**, **11**, and **12** (**Figure 3**) were incubated with the extracts of *P. reticulatum* and *K. brevisulcata*. Epoxidation was not detected, but the oxidation and hydrogenation of the aldehyde group of **3** were observed and the hydroxyl group of the dienol side chain of **10** was dehydrogenated. In addition, several unknown compounds derived from **9**, **10** and **12** were detected in the incubation with *K. brevisulcata*. Although a trace amount of the compounds derived from **10** were also detected in the incubation with *P. reticulatum*, the unknown reactions producing them were thought to be unique to *K. brevisulcata*. Furthermore, the reactions were presumed to involve isomerization that required an allylic hydroxyl or an amide group. The other unknown compounds were

observed in the incubation with *K. brevisulcata*, but their amounts were too small to confirm their identity.

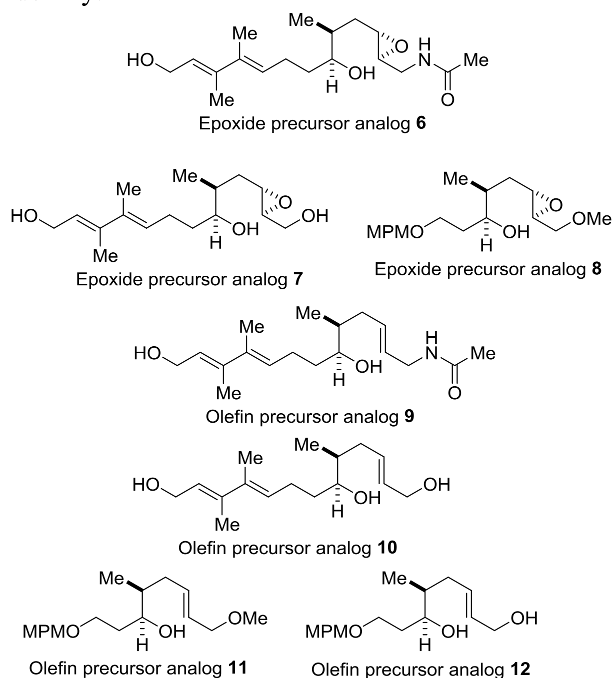

Fig. 3. Structures of synthesized epoxide and olefin precursors of brevisamide.

1-3 Aldehyde oxidase

When **2** was incubated with *P. reticulatum*, oxidation of the aldehyde group was observed. Since the oxidation occurred without addition of coenzymes such as $NAD(P)^+$, it was thought to be catalyzed by aldehyde oxidase (AO), which utilizes molecular oxygen as an electron acceptor, and not by aldehyde dehydrogenase which requires $NAD(P)^+$. If AO is indeed present it represents the first time AO has been found in dinoflagellates. Because of this it was examined in greater detail to determine whether AO represents a biosynthetic enzyme and if it has unique properties. Extracts of *P. reticulatum* were treated with acetone to facilitate precipitation and to remove small molecules. By using the resultant protein solution, the substrate specificity of AO was investigated. Several aromatic aldehydes, and (–)-citronellal (as an aliphatic aldehyde) were incubated with the AO containing solution. Oxidation of the aromatic aldehydes was not detected but oxidation of (–)-citronellal was observed. Therefore, AO found in this study was presumed to only have specificity to aliphatic aldehydes, and not to aromatic ones. A detailed study of AO will be performed once isolation of the enzyme is completed.

In the incubation experiments performed in this study using synthesized substrates (epoxides and olefins) with extracts of known polyether producing dinoflagellates, objective 6-*endo* cyclization and epoxidation was not detected. However, oxidation and hydrogenation of aldehyde groups, dehydrogenation of a hydroxyl group, and several additional unknown reactions that are presumed to be due to isomerization, were observed. Cyclic ether compounds produced by *Karenia* spp. such as brevetoxins, gymnocins (Satake *et al*. 2002, 2005; Tanaka *et al*. 2013) and brevisulcenal (Hamamoto *et al*. 2012) have enal side chain structures. Therefore, these types of reactions, especially the dehydrogenation of the hydroxy group, are potentially involved in the biosynthesis of ladder frame polyethers. The purification and characterization of the enzyme responsible for these reactions will be reported elsewhere.

Acknowledgements

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A noncryptic noncanonical multi-module PKS/NRPS found in dinoflagellates

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Abstract

Dinoflagellates produce a variety of compounds, likely using polyketide synthases (PKS) or non-ribosomal peptide synthases (NRPS) based on structure and labeling studies. An 8 domain hybrid PKS/NRPS greater than 8 thousand bases was identified in 19 deeply sequenced dinoflagellate transcriptomes. The gene was found in data from *Oxyrrhis marina* and 18 core dinoflagellates, but not the syndinean genus *Amoebophrya*. Spliced leaders, introns, and multiple subtle sequence variants were found, ruling out bacterial contaminants and confirming the dinoflagellate source of the sequences. However, the domain content and order are shared with a bacterial cryptic noncanonical PKS/NRPS (BurA) from *Burkholderia* spp., indicating a likely bacterial origin through horizontal gene transfer. The dinoflagellate and bacterial genes also have similar amino acid and malonyl CoA binding pockets. Based on labeling studies from *Burkholderia* spp. the dinoflagellates may use methionine to produce a propionate for incorporation into natural products.

Keywords: polyketide, non-ribosomal peptide synthase, dinoflagellate, *Burkholderia*

Introduction

Dinoflagellates produce unique polyketides (Van Wagoner *et al.* 2014) and are also known for adopting genes from bacteria via horizontal gene transfer (Butterfield *et al.* 2013, Rowan *et al.* 1996, Wisecaver *et al.* 2013). The primary proteins responsible for natural products in dinoflagellates are thought to be polyketide synthases (PKS) and non ribosomal peptide synthases (NRPS) or a hybrid of the two (Kellmann *et al.* 2010, Kubota *et al.* 2006, Lopez-Legentil *et al.* 2010, Murray *et al.* 2012). The PKS incorporate acetyl-CoA or malonyl-CoA using ketosynthase domains, often cyclically to produce structures of increasing size. For NRPS, amino acids are incorporated based on specificity defined by the adenylation domain. In hybrid PKS-NRPS both adenylation and ketosynthase domains are present. Processive multidomain PKS/NRPS proteins sequentially catalyze reactions in the order of the catalytic domains in the protein. In the simplest cases, the product of these sequential reactions can be predicted solely based on the arrangement in the genome. This has not proved to be true for dinoflagellates. Despite the remarkable complexity and variety of dinoflagellate chemistry, only a very limited group of precursor units are utilized to build these remarkable structures. They include only acetate, glycolate, and glycine (Van Wagoner *et al.* 2014).

In the search for potential natural product genes in dinoflagellates, typical queries include the conserved ketosynthase (KS) and adenylation domains (Kellmann *et al.* 2010, Kubota *et al.* 2006, Lopez-Legentil *et al.* 2010, Murray *et al.* 2012). Large mRNA-based sequence datasets for dinoflagellates demonstrated single domain KS are present in several different dinoflagellates, although adenylation domains have not been as well described (Rosic 2012, Lopez-Legentil *et al.* 2010). For example, transcripts were found in *Karenia brevis* containing the dinoflagellate hallmark spliced leader sequence followed by a single KS domain and phosphopantheinate domain (Monroe and Van Dolah 2008). A total of 7 KS domains and 1 ketoreductase domain were found in that Sanger sequence survey (Monroe and Van Dolah 2008). Similarly, a transcript containing a single KR domain and corresponding upstream genomic sequence without other PKS domains was determined for *Amphidinium carterae* (Bachvaroff and Place 2008), creating the notion that dinoflagellate PKS machinery contains only single module proteins. Similar results were later reported for *Hetercapsa triquetra* and *Alexandrium ostenfeldii* (Eichholz *et al.* 2012). In this study, the null-hypothesis that

	Amino acid binding pocket	Acyl Transferase binding residues
Burkholderia pseudomallei	NVWVFMAD	QQGHSIGRFHAHV
Amphidinium carterae	NVWLLCDD	QQGHSLGRFHRHV

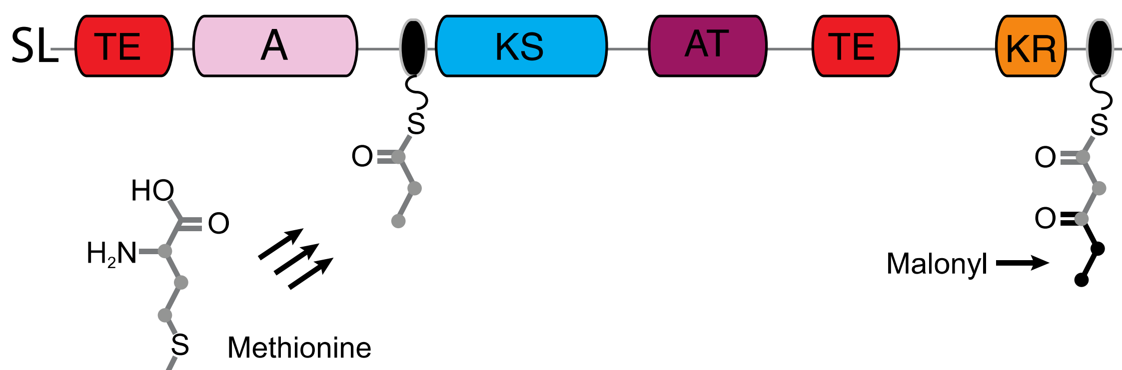

Fig. 1 Domain arrangement of the hybrid PKS/NRPS gene found in dinoflagellates. SL refers to spliced leader, TE to thioesterase, A to adenylation, KS to KetoSynthase, AT to acyltransferase, and KR to ketoreductase domains. The black filled domains are phosphopantheinate domains. The key amino acid binding residues from the adenylation and acyl transferase domains were extracted from one dinoflagellate and a bacterial sequence and are shown above the domain arrangement. The structures shown are based on reactions predicted to occur in *Burkholderia* spp.

dinoflagellates do not have PKS or NRPS arranged as multimodules was tested using large-scale mRNA datasets from 19 dinoflagellates.

Materials and Methods

Raw sequences were collected for 8 species from the 'Dinoflagellate Assembling the Tree of Life' project, as well as for 11 species from the 'Moore Foundation Microbial Transcriptome Sequencing' project (Bachvaroff *et al.* 2014, del Campo *et al.* 2014). Only species with multiple sequence datasets were included from the microbial transcriptome datasets. Sequences were assembled with CLC genomics (Qiagen, MD, United States) and Trinity (Grabherr *et al.* 2011), creating two independent assemblies for each taxon assembly.
The KS domains from *K. brevis* were used as BLAST queries against three 'exemplar' species, *Akashiwo sanguinea*, *Amphidinium carterae*, and *Karlodinium veneficum* (Monroe and Van Dolah 2008) and screened based on subject sequence length. The search was then expanded to the remaining dinoflagellates using the sequences from these three species as queries. Conserved domains were annotated using BLASTp and the PKS/NRPS finder software (Bachmann and Ravel 2009).
Iterative assembly was required for some species to assemble complete sequences. The top hits from the CLC genomics and the Trinity assemblies were combined using overlap consensus in Sequencher (GeneCodes, MI, United States) with manual editing of gaps interrupting the reading frame. Read mapping was done using bowtie2 (Langmead *et al.* 2009) and visualized with IGV (Thorvaldsdottir *et al* 2013) to guide overlap consensus assembly. Protein translations were generated using Sequencher or with perl scripts. Phylogenetic analysis was performed as described previously using the protein translation (Bachvaroff *et al.* 2014).
Genomic PCR was performed to identify introns using primers designed from the cDNA. The products were amplified and sequenced as previously described (Bachvaroff and Place 2008). Assembly of genomic sequence to the cDNA contigs was done in Sequencher with manual adjustment of intron boundaries.

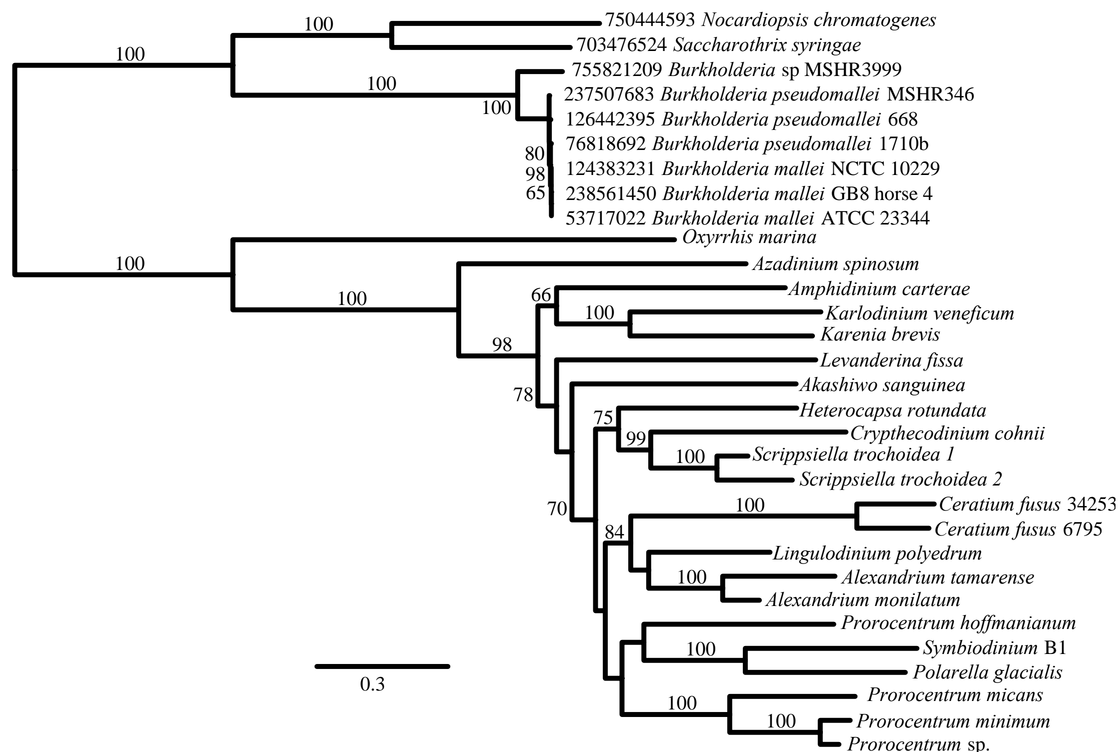

Fig. 2. A maximum likelihood phylogeny of the hybrid PKS/NRPS gene in dinoflagellates and bacteria, constructed using RAxML with the LG amino acid substitution matrix and gamma site to site rate variation. Bootstrap values from 100 replicates are shown when greater than 60 %.

Results and Discussion

A putative hybrid PKS-NRPS was found in all 19 dinoflagellate species (TreeBase 17476). In *A. carterae* this hybrid assembled into a single 8118 base contig, starting with a 13 base SL and an open reading frame of 7875 bases. Genomic amplification revealed introns of 78 and 471 bases across 142 bases of the cDNA.

In *A. sanguinea* the assembly of the apparent homolog was more complex, with polymorphism at both the nucleotide and amino acid level. Trinity assembled a single de Bruijn graph component into 16 individual sequences. Using overlap consensus in Sequencher, 10 of the sequences (ranging from 3984 to 257 bases) assembled into a single 9017 base contig. The remaining 6 sequences in this graph component were not part of the same contig based on read mapping. The consensus reassembly contained 331 polymorphic sites across an 8205 base open reading frame with 75 ambiguous amino acids in the consensus (2.7% amino acid polymorphism). A single Illumina sequence contained a 21 base SL sequence and was mapped near the 5´ end of the assembly, although the contig continued 223 bases upstream (5´ of) the putative SL acceptor AG identified by this single sequence. The CLC assembly of the same raw sequences yielded two contigs of 8412 and 9017 bases respectively. Together, the polymorphic Trinity contig and the two CLC contigs were 90 –94% identical at the amino acid level with a 9 amino acid length difference. Genomic amplification from *A. sanguinea* revealed no introns across approximately 5 kb of the cDNA. The apparent homolog from *K. veneficum* was a single Trinity contig, but lacked an SL sequence at the 5´ end. Genomic PCR was unsuccessful for *K. veneficum*. Based on the identical domain arrangement in *A. carterae*, *A. sanguinea,* and *K. veneficum*; an apparent homolog was identified from other dinoflagellates using BLAST. The BurA protein from *Burkholderia* spp. and other bacteria had identical domain arrangement (Franke *et al.* 2012). The *A. carterae* sequence and its bacterial counterparts were 35% identical across a full-length pairwise BLASTp alignment. The 8 PKS/NRPS domains (Fig. 1) included two thioesterase domains, one at the 3´ end, and a second nearer the 5´ end. In addition the amino acid binding pocket of the adenylation domain contained similar amino acids across the dinoflagellates and *Burkholderia* spp. (Stachelhaus *et al.* 1999). Similarly, the Acyl

Transferase domain contained amino acids in the binding pocket typical for malonyl-CoA binding (Khayatt *et al.* 2013).

Of the remaining 18 species, 16 assembled into a single contiguous open reading frame, although and CLC assembly from *Ceratium fusus* and *Scrippsiella trochoidea* yielded two full-length or near full-length contigs. In many cases, multiple high identity (but not full-length) sequences were present in the assembly, with differences as described above for *A. sanguinea*. These were condensed into a single consensus sequence for phylogenetic purposes. Overall these results suggest in most dinoflagellates multiple subtly variant gene copies are present in the genome, and transcripts were found over a diel cycle using QPCR (Williams *et al.* this volume).

The phylogenetic tree of full-length aligned BurA sequences is consistent with previous phylogenies of dinoflagellates (Fig. 2) (Bachvaroff *et al.* 2014). A few well-supported clades combine *Symbiodinium* sp. and *Polarella glacialis*, *K. veneficum* and *Karenia brevis*, three of four *Prorocentrum* spp., as well as *Alexandrium* with *Lingulodinium polyedrum* and *C. fusus*.

The presence of the BurA-like gene in a relatively broad sample of free-living dinoflagellates suggests acquisition prior to dinoflagellate diversification. In addition, the domain order and composition are conserved across both dinoflagellates and bacteria. The conserved domain order and overall similarity suggests the dinoflagellate and bacterial versions of the protein catalyze similar reactions. The bacterial BurA adds 3 carbons derived from methionine to malonyl-CoA. The major partner of BurA is BurF, another multidomain hybrid PKS/NRPS. However no obvious candidate partner has yet been found in dinoflagellates. The function of the dinoflagellate gene in metabolism remains obscure, but could likely provide 3 carbon propionate precursors via methionine.

Acknowledgements

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Mortality and pathology of Japanese scallop, *Patinopecten* (*Mizuhopecten*) *yessoensis*, and noble scallop, *Mimachlamys nobilis*, fed monoclonal culture of PTX-producer, *Dinophysis caudata*

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Abstract

Japanese scallops, *Patinopecten yessoensis*, and noble scallops, *Mimachlamys nobilis*, were fed monoclonal culture of *Dinophysis caudata*. Unexpectedly, scallops died. Preliminary experiments of the effects of *D. caudata* on the survival of *P. yessoensis* and *M. nobilis*, and on the histopathology of the hepatopancreas of a subset of moribund *P. yessoensis* were conducted. Hypersecretion of mucus and pseudofeces, decreased response to physical stimulus, paralysis, and then death were caused by *D. caudata* in both species. Within 1–7 days, 20–80% of Japanese scallops died. For *M. nobilis*, 20–60% died within 4–7 days. The mortality was significantly higher for Japanese scallops. The hepatopancreas of moribund *P. yessoensis* showed melanisation, myopathy, granuloma and lacunae. The strain of *D. caudata* fed to scallops produced only Pectenotoxin-2 (PTX-2), which is not lethal to bivalves. The PTX-2 cell quota of *D. caudata* fed to scallops was not correlated with the mortality of both scallops. This is the first evidence of lethal effects of a *Dinophysis* species to bivalve molluscs. The results of this study strongly suggest that the strain of *D. caudata* produces highly potent toxic compounds. Further investigative experiments are being conducted to characterize the toxin(s), and to determine the toxicity mechanism.

Keywords: Dinophysis caudata, DSP, *Patinopecten yessoensis*, *Mimachlamys nobilis*, Pathology, Mortality

Introduction

Several species of the genus *Dinophysis*, and to a lesser extent the benthic dinoflagellate *Prorocentrum lima*, are responsible for outbreaks of Diarrheic Shellfish Poisoning (DSP) in humans associated with the consumption of shellfish contaminated with Diarrheic Shellfish Toxins (DST). Following the recent removal of pectenotoxins (PTX) and yessotoxins (YTX), the DST regroup okadaic acid (OA)/dinophysistoxin (DTX) analogues (FAO 2011). The long-known principal toxins associated with DSP outbreaks in Japan, Europe, and North America are Dinophysistoxin-3 (DTX-3), Dinophysistoxin-1 (DTX-1), and Okadaic Acid (OA), respectively. After ingestion/digestion of the cells of *Dinophysis*, bivalve molluscs, the major vectors of DSP, are known to assimilate DST, in their original or less toxic modified forms, and later eliminate the toxins following a monophasic of biphasic kinetics described by one or two-compartment models (Silver and Cembella 1995). There are a few reports indirectly implicating DST in sublethal and lethal effects in aquatic organisms, although OA and YTX are lethal by injection into adductor muscle of scallops (Suzuki et al. 2005a). Detrimental or lethal effects following blooms of *Dinophysis* spp. or blooms/experimental exposure to *P. lima* have not been recorded. With the recent establishment of *Dinophysis* cultures (Park et al. 2006; Nishitani et al. 2008), experiments at our facilities were planned to study potential subtle to sublethal effects of *Dinophysis* spp. in bivalves, and to better address the kinetics of bioaccumulation/biotransformation of DST. Unpredictably, Japanese scallops, *Patinopecten yessoensis*, fed monoclonal culture of PTX–producer *D. caudata* died. Therefore, we conducted preliminary feeding experiments with *P. yessoensis*, and the noble scallop, *Mimachlamys nobilis*, to assess the mortality in both species, and investigated the potential presence of damage in hepatopanceras of *P. yessoensis*.

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Material and Methods

The monoclonal strain of *Dinophysis caudata* was isolated from Sumou-Nada, Nagasaki Prefecture, Japan (32.814°N, 129.763°E), in 2013. The culture of *D. caudata* was maintained at 21–23°C, in 250 mL capacity carbonate Erlenmeyer flasks, by feeding the marine ciliate, *Myrionecta rubra,* grown with the cryptophyte, *Teleaulax amphioxeia* (Nagai et al. 2004; Nishitani et al. 2008).The cell density of *D. caudata* from each culture flask were counted and 1 ml aliquots of *D. caudata* fed to scallops were sampled and preserved at –30°C until analyses of toxin content. Methanolic extracts from *D. caudata* cells were analyzed and quantitated by liquid chromatography-mass spectrometry (LC-MS) (Suzuki et al. 2005b, 2009).

Adult Japanese scallops (N=30, shell length=88.8±1.8 mm, wet weight=69.2±1.5 g) were collected from Hokkaido Island, Northern Japan, in Mai, August, and September 2014. After 2–3 weeks of acclimation, the shells of scallops were brushed and flushed with tap water to remove epibionts, and then washed in clean seawater. The byssus of noble scallops was cut before the start of the experiments. Japanese scallops were individually placed in 2 L-beakers (N=5, triplicate) filled with gently aerated natural seawater and fed a daily ration of $2{\times}10^5$ cells of *D. caudata*, at 15°C, for one week. Adult noble scallops, *Mimachlamys nobilis* (N=15, shell length=78.9±0.5 mm, wet weight=76.6±1.6 g) were shipped from Mie prefecture, Western Japan, in August 2014, and were subjected to the same treatment, at 18°C (N=5, duplicate). The experimental seawater was replaced every 48 h. The behavioral responses and cumulative mortalities were checked daily. At the end of the experiments, the hepatopancreas of moribund Japanese scallops were excised, fixed in 10% formalin-seawater solution, dehydrated, embedded in Paraplast Plus embedding medium, and 5-µm thin sections were stained with Hematoxylin/Eosin and observed with light microscopy.

Results and Discussion

Only pectenotoxin-2 (PTX-2) were detected (Fig. 1). The average cell quota of PTX-2 of *D. caudata* fed to both scallop species was 171.3±9.1 pg/cell (N=19). Control Japanese and noble scallops survived after one week under the experimental conditions and showed no signs of lethargy.

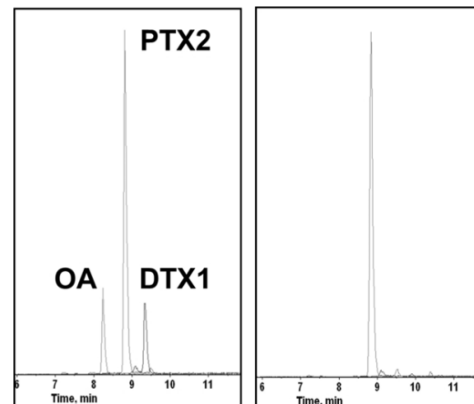

Fig.1. Chromatograms of toxin standards (left) and toxins detected in *D. caudata* cells (right). OA: okadaic acid, DTX1: Dinophysistoxin-1, PTX2: Pectenotoxin-2.

Mortalities of *P. yessoensis* fed $2{\times}10^5$ cells/day of *D. caudata*, varied between 20–80 %, within 24–72h of exposure, averaging 73.4±6.7 % after 1 week of exposure (Fig. 2).

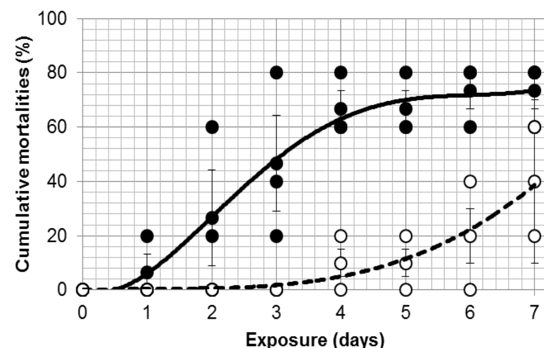

Fig.2. Mortality curves of Japanese scallops, *Patinopecten yessoensis* (red), and noble scallops, *Mimachlamys nobilis* (blue), fed daily a monoclonal culture of *Dinophysis caudata* ($2{\times}10^5$ cells)for one week. Bars: standard error of the mean, 95% CI.

For the same daily feeding ration, statistically lower mortalities of noble scallops, were observed within 96 h–168 h, and varied between 20–60%, averaging 40.0±20.0 % after 1 week (Fig. 2, Table 1). There were no significant differences in the

mortality between exposed replicas for each species of scallops, and between PTX-2 cell content of *D. caudata* fed to each replica of *M. nobilis* (Table 2); however, there was a significant difference in the PTX-2 cell content of *D. caudata* fed to each replica of Japanese scallops.

Table 1. Statistical analyses (ANOVA, Student's *T*-test) of the effects of *Dinophysis caudata* on mortalities of *Patinopecten yessoensis*, and *Mimachlamys nobilis*.

Scallop species	*P. yessoensis*	*M. nobilis*
Japanese vs. noble	$P < 0.05$	
Control vs. exposed	$P< 0.01$	$P< 0.05$
Replica	NS	NS
PTX-2 cell quota	$P< 0.001$	NS

NS: non-significant.

Japanese scallops exhibited altered behavior with hypersecretion of mucus and pseudofeces, retraction of the mantle and tentacles, reduced to suppressed escape response and delayed to suppressed response to physical stimuli, 24 h post-feeding (Table 2).

Table 2. Quantitative behavioral responses of *Patinopecten yessoensis*, and *Mimachlamys nobilis*, to *Dinophysis caudata*.

Scallop species	*P. yessoensis*			*M. nobilis*		
Duration of exposure (h)	24	48	72	24	48	72
Byssus production (%)	NA	NA	NA	50	20	0
Mucus and pseudofecal production	46.7	66.7	80	0	33.4	33.4
Delayed response to stimuli (%)	66.7	80	100	46.7	80	100

NA: not applicable.

All Japanese scallops became paralyzed following 3 days of feeding. The behavior of noble scallops was also affected but at lower rates. Hypersecretion of mucus and pseudofeces were observed in 1/3 of the scallops within 48 h, and the intensity did not increase further. Byssus was produced by 50 % of the scallops, 24 h post-feeding; however, after the second feeding only 20 % of scallops had byssus, and after the third feeding all scallops had lost their newly-produced byssus. The pseudofeces and fecal pellets of both noble and Japanese scallops contained intact, and intact and partially digested *D. caudata* cells, respectively (Fig. 3).

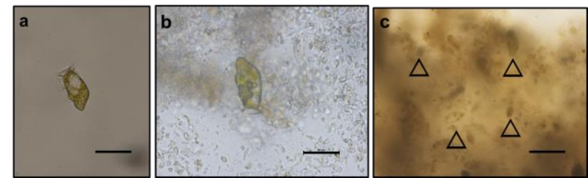

Fig. 3. *Dinophysis caudata* cells in (a) culture, (b) pseudofeces, (c) and in feces (arrow heads) of *P. yessoensis*. Scale bar: 50 µm.

The hepatopancreas of moribund *P. yessoensis*, showed severely necrotic connective tissue with lacunae, melanisation, myopathy with wavy muscle fibers, and granuloma (Fig. 4).

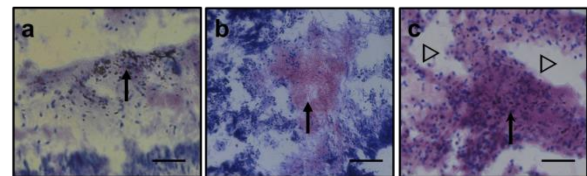

Fig. 4. Pathology in severely necrotic connective and epithelial tissues of the hepatopancreas of moribund *Patinopecten yessoensis* (N=3) fed *Dinophysis caudata* ($2{\times}10^5$ cells/day) for 1 week. (a) melanisation (black arrow), (b) myopathy (black arrow), (c) granuloma (black arrow) and lacunae (arrow heads). Scale bar: 50 µm.

Direct evidence of lethal effects of *Dinophysis* spp. in aquatic organisms has never been reported in the literature. Fish mortalities were reported in the Gulf of Thailand and the Seto Inland Sea during blooms of *D. caudata* (Okaichi 1967). In the summer of 1993, a large and long-lasting bloom of *D. caudata* (max cell density=$1.5{\times}10^3$ cells/mL) was reported in India, concomitant with a near-total absence of zooplankton, and a decline in the number of diatoms and fish (Santhanam and Srivasan 1996). In 1994–1995, mass mortalities of yellow clams, *Mesodesma mactroides*, and smaller quantity of cockles, *Donax hanleyanus*, were reported from the coasts of Uruguay near the Brazilian boarders (Méndez 1995). The plankton samples contained, along with several other dinoflagellates and diatoms, *D. caudata* ($4.6{\times}10^3$ cells/L) and *D. acuminata* ($3.6{\times}10^3$ cells/L); however, there was no direct evidence to incriminate both *Dinophysis* species in the mortalities of clams and cockles. Several species of bivalves collected during blooms of *Dinophysis* spp. or experimentally exposed to blooming waters of *Dinophysis* spp. are well documented to accumulate and transform DST during the

digestive processes, without being affected. In particular PTX-2, the only toxin from cells of the strain of *D. caudata* fed to scallops in this study, is hydrolyzed at higher rates than other PTX to PTX-2 seco acid (PTX2SA) (Suzuki et al., 2001; Torgsten et al. 2008). The scallop, *P. yessoensis*, however, lacks the ability to hydrolyze PTX and was shown to oxidize PTX-2 to PTX-6 mainly (Suzuki et al. 1998). The bioconversion of PTX-2 in noble scallops has not been studied, yet. The mortality of the scallops were not correlated with the PTX-2 content of *D. caudata*, and mortality of scallops due to PTX-2 has never been observed, neither in the wild nor under laboratory conditions. Japanese scallops directly injected with OA, YTX and PTX-6 in different organs showed mortality 9 days post-injection. These mortalities were related to a sudden increase in OA that would not mimic the natural uptake of OA. In addition, injection of PTX-6 caused lower mortality (Suzuki et al. 2005). In the same study, Japanese scallops that were injected with OA, potent inhibitor of protein phosphatase 2A and tumor promoter, showed hypersecretion of mucus and necrosis, but these pathologies were not observed in scallops injected with PTX-6. Therefore, the results of the present study are the first evidence of lethal effects of a *Dinophysis* species in marine bivalves. High mortalities of Japanese and noble scallops were caused by *D. caudata* monoclonal culture, under controlled laboratory conditions, through unknown toxins, other than PTX-2, or secondary metabolites.In this study, higher mortalities were reported for *P. yessoensis* than *M. nobilis* exposed to *D. caudata*. A cosmopolitan species, *D. caudata* is found in tropical and subtropical waters in mixture with other *Dinophysis* species, at moderate density ($<10^3$ cells/mL) with exceptionally high densities from tropical waters ($>10^6$ cells/mL). In Japan, it is mainly distributed in the west and occurs at very low densities in Hokkaido. Although at this stage, the causes underlying the difference in mortalities and responses of the two species are difficult to explain, it is possible that noble scallops, distributed in western Japan where densities and recurrence of *D. caudata* are higher than in Hokkaido, are less sensitive to the compounds associated with *D. caudata* than Japanese scallops. In conclusion, this study is the first report of behavioral alterations, pathologies in the digestive gland, and high mortalities in two species of scallops, *P. yessoensis* and *M. nobilis* fed monoclonal culture of *D. caudata*. The lethal effects were not associated with PTX-2, strongly suggesting the implication of unknown toxins and/or other metabolites. The results also suggest species-specific sensitivity to *D. caudata*; Japanese scallops being more sensitive than noble scallops. Further investigations are being conducted at our facilities to address both the toxins and the mechanism of toxicity.

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Developmental inhibition of zebrafish and sea urchin embryos by toxin producing *Ostreopsis* and *Gambierdiscus* isolates

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Abstract

In this study, the toxicity of *Ostreopsis* and *Gambierdiscus* micro-algae biotoxin containing extracts was assessed using zebrafish and sea urchin embryonic developmental bioassays. The *Ostreopsis* extracts tested were not toxic to zebrafish embryos with only one extract being toxic to sea urchin larvae. Maitotoxin and ciguatoxin producing *Gambierdiscus* strains were highly toxic to zebrafish embryos, whereas extracts containing putative maitotoxin-3 only were highly toxic in the sea urchin assay. These results show the sensitivity of these vertebrate and invertebrate model assays to micro-algal biotoxins and the variety of toxic responses that can be observed as a result of exposure.

Keywords: embryo toxicity, early life stages, bioassays, toxicity, marine toxins

Introduction

Epiphytic micro-algae of many genera have been shown to produce potent biotoxins. Some dinoflagellates of the genus *Ostreopsis* produce palytoxin (PLTX), ovatoxins, and associated analogues (Tubaro *et al.,* 2011) and species in the genus *Gambierdiscus* have been shown to produce ciguatoxins (CTX), maitotoxin (MTX) and analogues (Holmes and Lewis, 1994; Lewis, 2006).

Detection methods for these toxins from micro-algae and contaminated fish are continuously being developed (Tubaro *et al.,* 2014; Vetter *et al.,* 2014). The traditional mouse bioassay can produce false positive results as well as being ethically questionable (Campbell *et al.,* 2011), thus the need for new methods is a high priority.

Chemical methods including liquid chromatography paired with tandem mass spectrometry (LC-MS/MS) require known toxin standards and are not able to detect uncharacterised toxins. Embryonic *in vivo* bioassays have not been the focus of much biotoxin research, with preference being for *in vitro* models. *In vivo* models have an advantage in that they are not limited by particular mechanisms of toxicity, thus exhibiting a multi-faceted response to toxic material. Also, early life stages are often more sensitive to toxins than adult organisms (Vasconcelos *et al.,* 2010).

Here, vertebrate and invertebrate embryonic model bioassays were tested for their sensitivity to marine micro-algal extracts containing known biotoxins as determined by LC-MS/MS methods.

Material and Methods

Twelve micro-algae cultures from the Cawthron Institute Culture Collection of Micro-algae were grown and harvested at the stationary phase. Seven *Ostreopsis* and five *Gambierdiscus* strains were used (Table 1). Details of growth and extraction methods are summarised in Argyle (2014).

LC-MS/MS methods were used to determine the presence of palytoxin and/or related analogues from the *Ostreopsis* extracts using methods described by Selwood *et al.* (2012), and maitotoxin and ciguatoxins for *Gambierdiscus* extracts using methods from Kohli *et al.* (2014).

Zebrafish embryo toxicity (FET) tests were conducted following OECD test guideline 236 (2013). Briefly, four lethal end points were monitored at 24 hour intervals from 0 to 96 hours post fertilization.

Sea urchin fertilisation and development methods were conducted using experimental methods as derived from Fernandez and Beiras (2001) and Environment Canada (2011). In summary, fertilized eggs were exposed to dilutions of micro-algal extracts for 96 hours to observe effects on development of the embryos.

Table 1. Toxin production of each micro-algae strain tested.

Origin	Species	Toxin concentration (ng/mg dry weight)
Australia	*Ostreopsis sp.*	32.5 PLTX
New Zealand	*O. siamensis*	72.7 PLTX
New Zealand	*O. siamensis*	75.8 PLTX
Cook Islands	*O. ovata*	84.5 PLTX
Cook Islands	*O. ovata (?)*	-
New Caledonia	*Ostreopsis sp.*	165 PLTX
Cook Islands	*Ostreopsis sp.*	-
Cook Islands	*G. australes*	338.3 MTX/MTX-3*
New Zealand	*G. cf. yasumotoi*	MTX-3*
Cook Islands	*G. polynesiensis*	795.4 CTX/MTX-3*
Cook Islands	*G. pacificus*	MTX-3*
Cook Islands	*G. pacificus*	MTX-3*

*a putative MTX-3 compound detected

Results and Discussion

LC-MS/MS analysis showed five out of seven of the *Ostreopsis* isolates produced PLTX-like compounds. One *Gambierdiscus* produced MTX and one CTX, with all five producing a compound thought to be a putative MTX-3 like compound.

None of the PLTX-containing extracts caused any toxic response in the FET assay, indicating that the threshold of sensitivity to PLTX lies above the tested concentrations or that there is no adverse affect on the embryos. All *Gambierdiscus* extracts demonstrated some toxic response using the FET assay, with the MTX and CTX containing extracts being the most potent. The MTX-containing extract caused 100% embryo coagulation within 24 hours of exposure (Figure 1). This indicates an acute toxic effect through the chorion via passive exposure. Lower concentrations of this extract caused severe growth anomolies (Figure 2). The CTX-containing extract caused 100% spinal curvature in embryos, indicating a lack of somite formation (Figure 3).

The two Cook Islands MTX-3-containing extracts caused some abnormality in embryos (Figure 4), as did the New Zealand MTX-3-containing extract, but this was observed only in 5-10% of embryos across all concentrations tested.

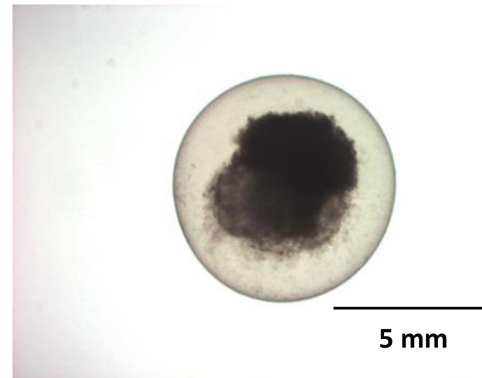

Fig. 1. MTX extract-treated embryo (1 mg/L) photographed at 24 hours post fertilisation, exhibiting coagulation.

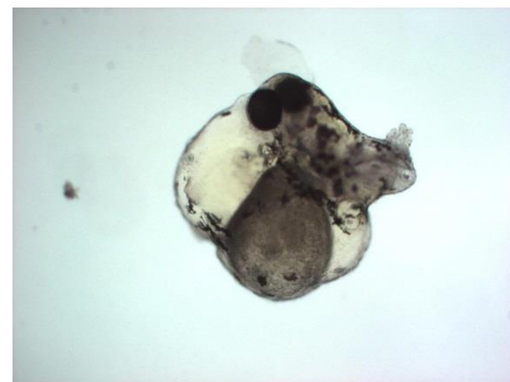

Fig. 2. MTX extract-treated embryo (0.31 mg/L) at 96 hours development with no detectable heartbeat.

Fig. 3. Detail of the spinal curvature exhibited by 1 mg/L CTX extract-treated embryos at 96 hours development.

Fig. 4. Cook Islands MTX-3 extract-treated embryo (0.095 mg/L) at 72 hours development showing distinct tail curvature indicating lack of somite formation.

The two Cook Islands MTX-3 extracts caused developmental inhibition in sea urchin embryos at 1 mg/L (Figure 5) and abnormalities at 0.56 mg/L including the growth of an additional arm (Figure 6). These results indicate an unknown toxic mechanism associated with extracts from these algae. It is not known if the putative MTX-3 compound is responsible for this response. However, as all *Gambierdiscus* strains tested to date produce this compound but not all exhibited this response, further investigation is required.

Fig. 5. Cook Islands MTX-3 extract-treated larva (1 mg/L) at 96 hours post fertilisation showing lack of development.

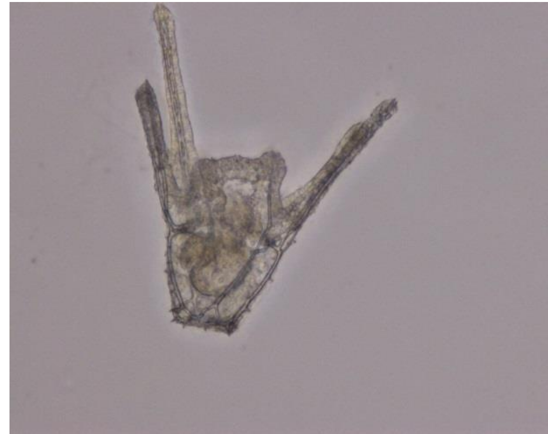

Fig. 6. Cook Islands MTX-3 extract-treated larva (0.56 mg/L) showing the growth of an additional long arm.

The MTX containing extract caused abnormal development in sea urchin larvae but not to the same extend as the Cook Islands MTX-3 extracts. The CTX-containing extract did not significantly inhibit development.

Larval development was also inhibited by the presence of one *Ostreopsis* extract from Noumea. This extract contained the highest level of PLTX-like compounds, indicating that the sea urchin assay is sensitive, but only to toxin levels higher than those in the other extracts tested.

Sea urchins are important grazers in tropical ecosystems, and *Ostreopsis* blooms have been connected to adult sea urchin mortality *in situ* (Shears and Ross, 2009). The results presented here suggest that early life stages of sea urchins may also be vulnerable to *Gambierdiscus* toxins, which could have implications for their reproductive success. Further research is needed to determine if such effects are observed *in situ* during *Gambierdiscus* blooms, and what the ecological consequences may be.

The effects of cigutera related micro-algae on fin fish is understudied, with a general assumption that fish are not negatively affected by the toxic cells they consume on the surface of macro-algae and corals. The results presented here show that larval fin fish are sensitive to micro-algal toxins through passive exposure, which could indicate that toxic micro-algal blooms might influence oviparous fish population dynamics.

This research showed that zebrafish and sea urchin embryonic bioassays may have use as

detection and monitoring tools for *Gambierdiscus* toxins. Further study is necessary to determine their sensitivity to *Ostreopsis* toxins, the mechanisms of toxic response, and to assess the potential ecological consequences of larvae mortality resulting from harmful micro-algae.

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Freshwater HAB Biology and Ecology

Ecophysiology of New Zealand *Didymosphenia geminata* nuisance diatom mats, compared to Tasmanian *Gomphonema* hydrofouling diatoms

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Abstract

The large stalk-forming diatom *Didymosphenia geminata* is responsible for nuisance blooms in New Zealand (NZ) streams and rivers, with negative impacts due to its massive production of persistent extracellular stalks. Because of these features the *D. geminata* problem has some similarities with the smaller stalk-forming *Gomphonema tarraleahae* biofouling issues in Tasmanian hydrocanals. Comparative nutrient and light adaptation responses were characterized by *in-situ* Pulse Amplitude Modulated (PAM) fluorometry on Buller River (NZ) *D. geminata*, Lake Rotoiti (NZ) *Gomphonema* cf. *manubrium* and Tasmanian *G. tarraleahae.* Although these stalk-forming diatoms had different requirements in terms of light, flow rate and nutrients, all three species were inhibited by silica addition and *D. geminata* was stimulated by iron. Due to its broad environmental tolerance, *D. geminata* if introduced would be likely to establish in Tasmanian hydrocanals, where environmental conditions are similar to New Zealand water courses where *D. geminata* blooms.

Key words: Didymospenia geminata, Gomphonema tarraleahae, nuiscance diatoms, New Zealand, Tasmania.

Introduction

The stalk-forming benthic diatom *Didymosphenia geminata* (Lyngbye) M. Schmidt is considered a nuisance, bloom forming species in New Zealand, representing one of the few examples of a potentially invasive freshwater microalga (Flöder & Kilroy 2009; Kilroy & Unwin 2011). Although *D. geminata* occurs in both running and standing waters (Spaulding & Elwell 2007; Kilroy & Bothwell 2011), nuisance blooms typically only occur in streams and rivers (Kilroy & Bothwell 2011; Root & O'Reilly 2012). They occur as extensive benthic mats, consisting of masses of cells and stalks that extend for greater than 1 km and persist for several months of the year. To the naked eye these mats resemble 'fiberglass insulation', 'tissue paper', 'rock snot', 'brown shag carpet' or 'sheep skin' covering the streambed (Spaulding & Elwell 2007; Fig. 1). Stalks can persist on substrates well after the death of the cells that produce them. This material comprises most of the biomass of algal mats, and is responsible for the negative impact of *D. geminata* (Kirkwood *et al.* 2007). Blooms of *D. geminata,* in contrast to many other algal blooms,
are associated with low-nutrient waters (Spaulding & Elwell 2007). These aspects of *D. geminata* compare to the nuisance diatom *Gomphonema tarraleahae* Perkins and Hallegraeff in Tasmanian hydrocanals (Perkins *et al.* 2009).

Fig. 1 *Didymosphenia geminata* stalk material from Buller River, New Zealand, as it appears when out of water (a) and covering stream bed

Biofouling in the Tarraleah hydro-canals represents an estimated 1.8-17.6 tonnes (dry weight) of fouling in the 20 km long canal (Perkins *et al.,* 2009), causing up to 10% reductions in flow carrying capacity (Andrewartha et al., 2010). In the present study nutrient bioassays using PAM fluorometry were applied to allow instantaneous assessment of

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nutrient status of Buller River (NZ) *D. geminata*, Lake Rotoiti (NZ) *Gomphonema* cf. *manubrium* Fricke and Tasmanian *G. tarraleahae*.

Methods

Field samples of *D. geminata* and *G.* cf. *manubrium* were collected on 31 October 2013 respectively from the Buller River (41°47'11.5"S 172°48'44.5"E) and Lake Rotoiti (41°48'24.2"S 172°50'37.9"E), NZ and of *G. tarraleahae* on 11 December 2013 from Tarraleah (Tasmania) hydro-canals (42°18'29.4"S 146°25'24.4"E) by gently scraping of rocks, pylons and canal walls, a few centimetres below the water surface. For each species five plastic containers were prepared with 45 mL of river, lake and canal water. Nutrients (0.25 mL) were added to each container as follow (Table 1):

Table. 1. Final concentrations of nutrients applied in the nutrient bioassays

Container	Nutrient
Control	nothing added
Silica (Na_2SiO_3)	Si [36 mg/l]
Nitrogen ($NaNO_3$)	N [77 mg/l]
Phosphorus (K_2HPO_4)	P [8 mg/l]
Iron ($C_{10}H_{12}N_2NaFeO_8$)	Fe [0.00152 mg/l]

The content of each container was then divided in three equal fractions, in order to obtain triplicate treatments. A section of mat was added to each container. Containers were then placed in the dark at ambient temperature for 1 h before the first fluorescence measurement was performed in the cuvette of a Water-Pulse Amplitude Modulated (PAM) fluorometer (Waltz, GmbH, Effeltrich, Germany; gain setting 5–25). Samples were then returned to the dark for 3.5 h, followed by a second fluorescence measurement. Maximum quantum yield Fv/Fm (representing physiological health and influenced by nutrient stress) and rapid light curves (RLC) were assessed, as indicators of physiological health. Light curves were constructed by plotting average relative electron transport rate (rETR) against photosynthetically active radiation (PAR).

Results

With *D. geminata* both rETR and Fv/Fm values decreased after 1 and 4 h dark adaptation, in response to silica addition when compared to controls and rETR was reduced by 50 and 60%, respectively. Nitrogen addition after 1 h dark adaptation induced rETR to increase above the control (129 and 118, respectively) (Fig. 2), whilst after 4 h rETR was above control only in response to iron addition (data not shown). The highest Fv/Fm value after 1 and 4 h dark adaptation (0.59-0.55) was achieved after phosphorus addition and in general all Fv/Fm values decreased after 4 h of dark adaptation (Table 2).

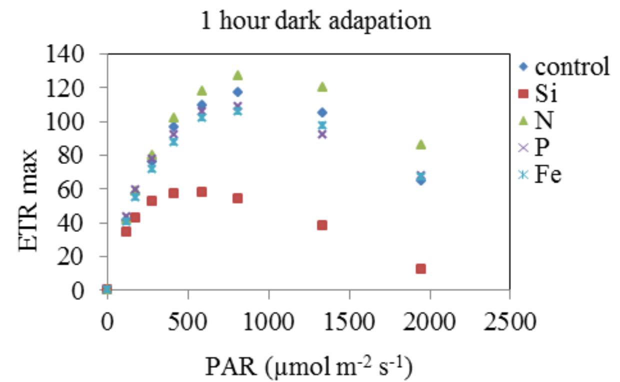

Fig. 2 *Didymosphenia geminata* rETR in various nutrient bioassays after 1 h dark adaptation

G. cf. *manubrium* showed a similar response to *D. geminata*, with rETR depressed in response to silica addition, in comparison to the control, both after 1 (from 89 down to72) and 4 h (from 93 down to 69) of dark adaptation. Iron addition caused rETR to increase above the control and other treatments, especially after 4 h dark adaptation (data not shown). Fv/Fm was above control in all treatments, both after 1 and 4 h dark adaptation (Table 2).

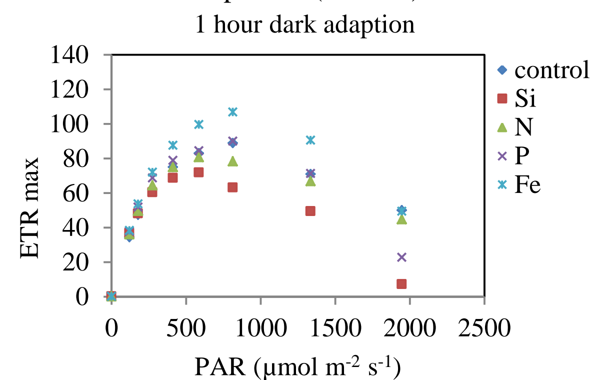

Fig. 3 *Gomphonema* cf. *manubrium* rETR in various nutrient bioassays after 1 h dark adaptation

With *G. tarraleahae* silica supply induced rETR to decrease both after 1 and 4 h dark adaptation of 40 and 25% respectively compared to the control. The other rETR values were similar to the control after 1 h (Fig. 4) and only iron addition depressed values by 10% after 4 h dark adaptation (data not shown). All Fv/Fm values were high (>0.7) after 1 h dark adaptation. After 4 h, phosphorus treatment showed the lowest Fv/Fm but still considered high for phytoplankton (> 0.6) (Table 2).

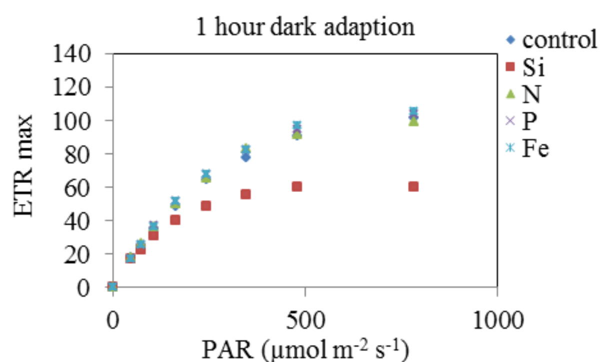

Fig. 4. *Gomphonema tarraleahae* rETR in various nutrient bioassays after 1 h dark adaptation

Discussion

The New Zealand diatom species *D. geminata* and *G.* cf. *manubrium* and the Tasmanian *G. tarraleahae* exhibited similar responses to silica addition, which in all cases surprisingly depressed their fluorescence rETR responses and in *D. geminata* also depressed Fv/Fm.

The role of silica in the formation of the diatom frustule is well known, and therefore the fluorescence Fv/Fm and rETR decrease in response to silica addition was unexpected.

Table 2. Fv/Fm values for *Didymosphenia geminata*, *Gomphonema* cf. *manubrium* and *Gomphonema tarraleahae,* in response to silica, nitrogen, phosphorus and iron addition, after 1 and 4 hr incubations

Species	Dark adapted	Control	Si	N	P	Fe
D. geminata	1 h	0.57±0.01	0.43±0.03	0.56±0.02	0.59±0.04	0.56±0.02
	4 h	0.53±0.01	0.35±0.04	0.53±0.01	0.55±0.01	0.54±0.03
G.cf.manubrium	1 h	0.44±0.04	0.50± 0	0.52±0.01	0.50± 0	0.50±0.01
	4 h	0.44±0.04	0.50±0.01	0.51±0.01	0.50± 0	0.50±0.01
G. tarraleahae	1 h	0.70±0.09	0.78±0.03	0.76±0.02	0.73±0.02	0.72±0.01
	4 h	0.74±0.02	0.74±0.01	0.72±0.02	0.62±0.08	0.66±0.04

Lippemeier *et al* (1999; 2001) reported a decrease in Fv/Fm due to silicate starvation in cultures of *Thalassiosira weissflogii*. Decreased protein synthesis occurs in silicon-starved cells, leading to reduced repair of damaged PSII centres causing in turn a reduction in Fv/Fm. A possible explanation for our results relates to the application of unrealistically high silica concentrations (36 mg L^{-1}) to these diatom species all adapted to oligotrophic waters (0.95-4.8 mg L^{-1} silica content for Tarraleah No.1 canal; 1.82-2.28 mg L^{-1} for Buller River, average between two sites from Oct 2013 to March 2014). Iron addition after 4h triggered an increase in fluorescence only in *D. geminata* and *G.* cf. *manubrium*, but not for Tasmanian *G. tarraleahae* (data not shown). Considering that the photosynthetic light harvesting and energy transduction apparatus are driven by proteins which bind iron atoms to mediate electron transfer, a positive fluorescence response to iron addition could be explained by a

lack of this critical micronutrient (75-118 µg L^{-1} in Tarraleah; 0-19.97 µg L^{-1} for Buller River, average between two sites from Oct 2013 to March 2014). According to Sundareshwar *et al.* (2011), mucopolysaccaride stalks in *D. geminata* play a role in nutrient adsorption because soluble iron can be absorbed from the stalks in the oxidized surface layer where, due to strong affinity, it binds phosphorus which is not bioavailable. Through an abiotic process phosphorus then becomes available in the inner layer of the mat, at a concentration at least an order of magnitude greater than present in surface water. Iron reactivity and concentration have thus been implicated as key factors determining *D. geminata* distribution. These processes may create a positive feedback between stalk biomass, which forms under low phosphorus, and cell division rates, which occurs in phosphorus-replete conditions. This concept matches that suggested by Cullis *et al.* (2012) claiming that the key to the *D. geminata* paradox (high biomass, low nutrients) is the temporal separation between mat growth and cell division, driven by nutrient content of the water. Under high light levels, in low nutrient conditions stalk production is high and cell division low, whilst when nutrient content increases cell division becomes predominant (Kilroy & Bothwell 2011). In contrast, Bothwell *et al.* (2012) suggested that *D. geminata* blooms can only occur in waters with low phosphorus- and iron content and that iron is not

responsible for promoting or sustaining the blooms in phosphorus-depleted waters. Whilst the role of phosphorus- and iron in *D. geminata* blooms is still debated, our results show a short term positive response of *D. geminata* to iron and this requires further investigation at more sites and with varying concentration of iron. Even though the parameters that define the habitat for *D. geminata* are not yet fully understood, its temporal and spatial distribution suggest a range of potential factors, including light availability, play important roles (James *et al* 2014; Whitton et al 2009). In contrast to *D. geminata*, the Tasmanian *G. tarraleahae* has a preference for low-light. In Tarraleah No. 1 canal fouling prevails on the northern wall which is more shaded by the vegetation (Perkins *et al* 2009). Though the general hydraulic preference for *D. geminata* blooms includes relatively low, stable flows (Kilroy 2004; Spaulding & Elwell 2007), a high degree of variation was found in *D. geminata* presence or absence relative to flow velocity (Kirkwood *et al* 2007). In contrast, the Tasmanian *G. tarraleahae* distribution is limited to high velocity areas (water velocity in Tarraleah No. 1 canal has an average of 2.1 m/s, with maximum flow rate of 24 m^3/s). In conclusion, due to its broad environmental tolerance, if introduced, *D. geminata* is likely to establish in Tasmanian Hydro Canals, because the overall conditions are similar to those encountered in New Zealand water courses.

Acknowledgements

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Lake phytoplankton assemblages; exploring the role of environmental and species interactions with cyanobacteria populations

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Abstract

Cyanobacteria are often considered one of major threats to lake health due to their toxin and bloom forming features. In this study, a multivariate non-parametric analysis (Vector Generalized Additive Model) was applied to investigate the relationship between abiotic and biotic factors and the growth of cyanobacteria populations in seven Auckland lakes. Our results suggest that within the epilimnion ecosystem there are strong inter-specific competitive relationships between different phytoplankton groups. Which species dominate the ecosystem depends upon both the abiotic environment and the presence of other species. Ochrophyta (mostly Bacillariophyta), Chlorophyta and Dinophyta were found to be strong competitors for phosphorus in comparison with Cyanobacteria. Addionally, abiotic factors such as turbidity and the ratio between total nitrogen and total phosphorus (TN/TP) have a strong relationship with the variation of Cyanobacteria in most of the study lakes. However, nitrogen is plentiful in most of the study lakes and we propose that this leads the environment to favour non-nitrogen fixing Cyanobacteria species, such as *Microcystis*.

Keywords: Cyanobacteria, lake water quality, TN/TP ratio, Vector Generalized Additive Models

Introduction

Blue-green algae (Cyanobacteria) are commonly found in lakes and can present a problem for management authorities, due to their potential to form toxic blooms. To achieve effective management action, good forecasting models are required to predict development of Cyanobacteria blooms. In Cyanobacteria dominated eutrophic lakes, population increase shows a strong relationship with nutrient input (Downing et al., 2001; Schindler, 1977; Havens et al., 2003). However, for non-Cyanobacteria dominated lakes, growth of Cyanobacteria populations is more complex and the factors affecting such growth is the focus of the research reported here. In the Auckland region, the seven largest lakes have been monitored since 2002, for their abiotic and planktonic characteristics. The water quality of these lakes ranges from mesotrophic to supertrophic (Barnes & Burnes, 2005); the catchments varying from partly forested, residential to intense pastoral farming. Normally these lakes are considered to be non-Cyanobacteria dominated; however, in the past there have been occasional bloom events in some lakes (Auckland regional public health service, 2006). Seven phyla of phytoplankton algae are commonly encountered in Auckland lakes: Chlorophyta, Euglenophyta, Ochrophyta, Dinophyta, Charophyta, Cryptophyta and Cyanobacteria. In the seven monitored lakes, assemblages tend to be dominated by species from Chlorophyta, Bacillariophyta, Cyanobacteria and Dinophyta groups.

Material and Methods

Sampling method and locations

Epiliminion phytoplankton and water quality data were collected by the Auckland Council (AC) lake management group following a standard sampling methods (AC sampling protocol, 2012). Monitoring commenced in 2002 and continues to the present day. Water and biological samples were collected six times per year during August, mid November, mid January, late February, mid April and late May. Samples were taken from within the photic zone, at the mid point of the epiliminion zone and at the deepest part of the lake (AC sampling protocol, 2012).

Data analysis

Initially standard parametric regression techniques were tried; however, most of the data does not conform to the requirements of parametric tests. Instead we have used a non-parametric approach,

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specifically Vector Generalized Additive Models (VGAM) (Yee and Wild, 1996) as implemented in R (Ihaka and Gentleman, 1997).

VGAM. VGAM is an extend version of the common non-parametric approach Generalized Additive Models (GAM) (Hastie & Tibshirani, 1990). VGAM uses vector smoothing spline functions, which are an extension of cubic smoothing functions. The advantage of this approach is that it offers an improved model analysis, by adding accuracy in defining the fit without over-smoothing, i.e. VGAM is a strongly data driven approach (T. Yee, personal communication, 20 September, 2014). All the data was standardized with a log (1+x) function. The beta version of VGAM (0.9-5) provides a VGLM (Vector Generalized linear Model) function to help identify the candidate variables for a full VGAM. Initially, all the biotic and abiotic variables were included in a VGLM analysis for a comprehensive candidate variable investigation. This enables an iterative selection process to be carried out, by deleting at each step the weakest explanatory variable. The process was repeated until all the variables in the model were determined to have significant relationships with Cyanobacteria (e.g. all the p-values are smaller than 0.01).

VGAM model analysis. In the analysis a unique spline function is developed for each explanatory variable, in the context of the other variables in the model. The model fitting process of VGAM changes the effective degrees of freedom to adjust the level of smoothing (Yee and Wild, 1996). A good fit is achieved by minimizing error, whilst avoiding over-fitting, i.e. a balance between under-smoothing and over-smoothing. The final model is tested by running a correlation between predicted and measured values. Initially a model based on the combined data from all lakes at each sampling period was developed. However, this approach only provided a weak predictive capability. Subsequently, a separate model was developed for each lake.

Results and Discussion

Core phytoplankton characteristics of the lakes

Table 1 illustrates the median biovolume ($mm^3\ l^{-1}$) of the major phytoplankton phyla in the studied lakes. Over the period of study (2002-2011) Cyanobacteria did not normally dominate any of the lakes. The median biovolume of Dinophyta, Ochrophyta and Chlorophyta was typically much higher than Cyanobacteria. Five of the seven lakes were commonly dominated by Dinophyta; however, this can be temporally variable. For example, during 2004-2007 lakes Kuwakatai and Spectacle were intermittently dominated by Cyanobacteria.

Water quality characteristics

Table 2 sets out the median water quality characteristics for the seven lakes over the period 2002-2011. Lake Spectacle can be considered super-eutrophic, whereas the other lakes show a mix of eutrophic and mesotrophic conditions (Burns et al., 2000).

Table 2. Median biovolume ($mm^3\ l^{-1}$) of major phytoplankton phyla in the lakes for the period 2002-2011. Highlighted cells indicate which phyla were important for the prediction of Cyanobacteria population size

	Charophyta	Chlorophyta	Cryptophyta	Cyanobacteria	Dinophyta	Euglenophyta	Ochrophyta
Kereta	0.0768	1.1071	0.0777	0.0036	0.000	0.0031	0.1444
Kuwakatai	0.4380	0.0499	0.0082	0.1320	16.0800	0.0659	0.1014
Ototoa	0.0185	0.0255	0.0071	0.0019	4.3444	0.0067	0.0315
Pupuke	0.4087	0.0506	0.0090	0.0207	6.6000	0.000	0.1852
Spectacle	0.0000	0.5843	0.0180	1.0780	0.0000	0.3397	15.0760
Tomarata	0.0094	0.0427	0.0068	0.0004	2.8152	0.0021	1.0193
Wainamu	0.0984	0.0206	0.0014	0.0000	8.4000	0.0976	0.1359

Table 3. Median concentrations of the measured water quality variables for the period 2002-2011. The highlighted cells indicate which variables were important for the prediction of Cyanobacteria population size.

Variables/Lake	Kereta	Kuwakatai	Ototoa	Pupuke	Spectacle	Tomarata	Wainamu
TN (mg/L)	0.69[b]	0.71[b]	0.25[c]	0.28[c]	1.2[a]	0.45[b]	0.32[c]
TP (mg/L)	0.03[b]	0.04[b]	0.02[b]	0.02[b]	0.09[a]	0.02[b]	0.03[b]
TN/TP	20.62	16.73	18.42	22.65	12.85	24.13	11.28
Turbidity (NTU)	2.40	3.97	0.74	0.84	19.4	1.70	5.46
pH	8.35	7.97	7.74	8.47	7.53	7.50	7.60
Dissolved Oxygen (ppm)	10.90	8.80	8.70	9.00	9.10	8.53	8.60
Soluble P (mg/L)	0.01	0.01	0.01	0.01	0.01	0.01	0.03
Water Temperature (°C)	20.75	18.67	18.95	19.15	19.50	19.40	18.35
Chlorophyll *a* (mg/L)	0.00	0.03	0.00	0.01	0.05	0.01	0.01
Suspended Solids (mg/L)	4.05	5.00	1.35	1.63	20.00	2.00	3.60

Notes. [a] indicates super-eutrophic; [b] indicates eutrophic; [c] indicates mesotrophic

Data analysis

A VGLM analysis for a regional lake model indicated that for the data available, no regional model can be developed. This suggests the variation of Cyanobacteria populations depends upon the local conditions at each lake; consequently each lake has been analysed separately. Follow up, lake specific VGAM analyses reinforced the role of local lake conditions on variation in Cyanobacteria populations. The combinations of abiotic and biotic variables that explain the population size of Cyanobacteria are indicated in Tables 1 and 2. Among the abiotic variables, turbidity and the various measures of phosphorus and nitrogen were the most common predictors of population size. In some studies, water temperature has been associated with the onset of Cyanobacteria blooms (Kosten, et al., 2012); whereas in this study, water temperature appeared to have only a minor role. Variation in abundance of some phytoplankton phyla, especially Ochrophyta, was found to show a strong relationship with variation in Cyanobacteria population levels. However, the nature of this association varied between lakes. Lake Ototoa was the only lake in which biotic variables were not significant predictors of Cyanobacteria population levels.

The results from the VGAM modelling approach, suggest good predictive models can be developed for each lake (Table 3). Although in the case of Lake Ototoa (the most oligotrophic lake), the fitted model was not as good.

Table 4. Comparison of predicted and actual values using the VGAM models for each lake. r is the correlation coefficient which measures the correlation relationship between predicted Cyanobacteria volume and measured volume. A p-value with less than 0.001 indicates the significance of the result.* indicates the lake stratifies over summer.

	Wainamu*	Tomarata	Spectacle	Pupuke*	Kuwakatai*	Kereta	Ototoa*
r	0.74	0.92	0.92	0.83	0.87	0.89	0.57
p	$\leq$0.001	$\leq$0.001	$\leq$0.001	$\leq$0.001	$\leq$0.001	$\leq$0.001	$\leq$0.001

Discussion

These results suggest, that to understand variability in Cyanobacteria populations in these lakes, requires knowledge of both the biotic and abiotic environment. Within the epilimnion ecosystem there are probably strong inter-specific competitive relationships in action. Which species ultimately come to dominate the ecosystem will depend upon both the abiotic environment and the presence of other species. In the summer, the study lake group comprises both stratified and unstratified lakes. It is unclear at this time as to whether any significance can be placed on the best models being developed for the shallowest, non-stratifying lakes. Overall this result suggests that for most of the time, even though most of these lakes are nutrient enriched, other phytoplankton phyla are out-competing Cyanobacteria. This result is similar to those studies that have suggested that Cyanobacteria may be poor competitors for phosphorus in comparison with some Ochrophyta (mainly Bacillariophyta) and Chlorophyta species (Tilman et al., 1986; Grover, 1989; Hu and Zhang, 1993). In our study lakes, Ochrophyta (mostly Bacillariophyta) and Chlorophyta are a major part of epilimnion phytoplankton assemblage and are correlated with cyanobacteria population size. In the lowest trophic level study lake (Ototoa), phytoplankton communities did not appear to show any relationship with Cyanobacteria levels. This may give support to the competition hypothesis, in that in this lake all phytoplankton may be nutrient limited. A TN/TP ratio of <16 has been widely used as an indicator of risk of Cyanobacteria bloom development (Schindler, 1977; Smith, 1983). Four of the seven lakes in general show a TN/TP ratio of close to or less than this value (Table 2). If this relationship holds for these lakes, then it suggests that there is a strong risk of Cyanobacteria blooms for at least four of the lakes. However, the Auckland lakes tend to be enriched with nitrogen rather than phosphorus and so would tend to favour non-nitrogen fixing Cyanobacteria such as *Microcystis*. This has been observed to be the case for Lake Kuwakatai when Cyanobacteria blooms formed in the past. Hence for the Auckland lakes, the TN/TP ratio is of limited value for prediction of Cyanobacteria blooms.The dual control of both N and P has become important in the management of lakes in Auckland, as it has been shown that both N and P can potentially increase the risks of Cyanobacteria blooms.

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Production of Siderophores by Freshwater Cyanobacteria in the Lower Laurentian Great Lakes

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Abstract

Cyanobacteria generally have higher iron requirements than most eukaryotic algae, and culture studies have shown that some cyanobacteria produce strong ferric iron chelators (siderophores) to facilitate iron uptake under iron-limited conditions. However this mechanism has not been well characterized in natural systems, due largely to limitations in methodology. To evaluate the importance of siderophores to natural plankton assemblages in the Laurentian Great Lakes, a novel solid-phase extraction was developed using high capacity C18 and polymeric cartridges and used for shipboard extractions on samples collected across a range of trophic conditions and cyanobacterial densities. A total of 70 samples were collected from Lake Erie (10 sites) and Lake Ontario (8 sites) between 2012-2014. Eluents from the SPEs were later analyzed for hydroxamate siderophores using two methods: the Csáky test and liquid chromatography-mass spectrometry. Although many of the sites were dominated by cyanobacteria, *in situ* production of hydroxamate siderophores was detected at only one highly eutrophic site in Lake Erie (Sandusky Bay). These results suggest that plankton at offshore sites in Lake Erie and all locations in Lake Ontario were not iron limited, that non-hydroxamate siderophores were produced, or that the biomass was insufficient to generate detectable levels of iron-binding compounds.

Keywords: cyanobacteria, Great Lakes, nutrient limitation, siderophores

Introduction

Cyanobacterial blooms are a global issue and a growing problem in the lower Laurentian Great Lakes, especially in the western basin of Lake Erie and several embayments of Lake Ontario (Downing *et al*. 2001; Watson *et al*. 2008; Steffen *et al*. 2014). Most studies have examined the importance of macronutrients (nitrogen (N) and phosphorus (P) on the formation and maintenance of cyanobacterial blooms (Steffen *et al.* 2014). A smaller number of studies have focused on the importance of micronutrients, particularly iron (Fe) (Twiss *et al*. 2000, 2005; North *et al*. 2007; Molot *et al*. 2014). The bioavailability of iron may be limited in oxic waters, where most extracellular Fe is in the form of iron colloids or bound to particulate or low molecular weight dissolved organic matter. Dissolved Fe (defined as Fe which passes through an 0.2 μm filter) is generally low, ranging between 2 – 34 nM in the surface waters of Lake Erie (Twiss *et al*. 2000, 2005). These concentrations are significantly higher than the total Fe measured in the open ocean (50 – 150 pM, Roy *et al*. 2008) and there has been debate if the offshore waters of the Laurentian Great Lakes are limited by trace nutrients (Twiss *et al*. 2000, 2005). Several studies (Nagai *et al*. 2007; Dang *et al*. 2012) have suggested the iron quota for bloom-forming *Microcystis aeruginosa* ranges from 1 – 60 fmol Fe/cell. If so, then the concentration of bioavailable iron needed to support a bloom of 10^9 cells/L would be approximately 1 nM. These concentrations may not be available in the offshore waters of the Great Lakes.

Some cyanobacteria produce Fe-chelating biomolecules called siderophores in iron-limited cultures to assist with iron acquisition (Goldman *et al*. 1983; Hutchins *et al*. 1991, Wilhelm and Trick 1994; Kranzler *et al*. 2013). Siderophore formation has been mainly associated with N-fixing species, as this activity increases the cellular iron requirement (Rueter 1988; Berman-Frank *et al*. 2007). It has also been observed in cultures of some non-diazotrophic species (Wilhelm and Trick 1994). Detection of siderophores in freshwater environments under actual bloom conditions has proved to be much more difficult than in laboratory cultures. Murphy *et al*. (1983) detected low molecular

weight iron chelators in a eutrophic lake in British Columbia, Canada. More recently, catechol and hydroxamate groups, potentially from siderophores, have been detected in oligotrophic lakes in the Algoma Highlands, Canada (Sorichetti *et al*. 2014). One issue may be that low iron concentrations are often associated with low nutrient concentrations, and siderophore formation and N-fixation may be mutually exclusive processes under these oligotrophic conditions (Hutchins *et al*. 1991; Berman-Frank *et al*. 2007). For this reason, the production of siderophores in low-iron high-nutrient (i.e. N, P) cultures may not be a good indicator of the importance of these chelators in natural planktonic communities. One common method for the collection and concentration of siderophores uses solid phase extraction cartridges (SPEs). Absorption to C18 SPEs have been shown to enrich siderophores from both saltwater and freshwater environments (Freeman and Boyer, 1992). SPEs with varying functional groups have been used to isolate Fe-chelating compound from marine environments (Donat *et al*. 1983; Boiteau *et al*. 2013). These studies all required the collection of large volumes of water to enrich the trace levels of siderophores present in those environments. Here we develop a similar enrichment technique for siderophores formation in freshwater systems using C18 and polymeric solid phase extraction cartridges.

Materials and Methods

Preliminary studies used solid phase extraction cartridges (SPEs) from various manufactures including a Waters C18 (10 g), Phenomenex C18 (10 g), Phenomenex C18 (20 g), and Phenomenex Strata-X (5 g) to determine their optimal volume and recovery. Each cartridge was equilibrated with methanol, rinsed with water, and ferrated desferrioxamine-B (DFB) was passed through the SPE in 1-20 L of distilled water acidified to pH ~ 3 using formic acid. Elution of the DFB followed the same procedure described for field samples below, and the DFB was quantitated in the eluent using high performance liquid chromatography with photodiode array detection (HPLC-PDA). To identify where potential losses could occur, the procedure was run stepwise in reverse using a known amount of DFB added at each step. Field samples were collected aboard the Canadian Coast Guard Ship *Limnos* from May-October of 2012-2014 on Lakes Erie and Ontario (Figure 1). Twenty liters of water were collected from a depth of 1 m using a submersible pump, and filtered through a 9 cm 934-AH filter (Whatman, 1.5 µm pore) using a peristaltic pump and 9 cm stainless steel filter holder. The filtrate was acidified to pH ~ 3 with formic acid and passed through a high capacity SPEs (Waters C18, 10 g in 2012 and 2013; Phenomenex Strata-X, 5 g in 2014) using a peristaltic pump. The SPEs were primed with 50 mL methanol followed by 50 mL distilled water prior to use. Once filtrate had passed through the SPE, the SPE was washed with 50 mL distilled water and frozen at -20 °C until elution. The cartridges were brought to room temperature in the laboratory and eluted with 50 mL methanol into a round bottom flask containing 50 mL distilled water. The methanol was removed *in vacuo* using a rotary evaporator and the remaining water removed by lyophilization. Once dry, the contents were reconstituted in 3 mL of distilled water and stored at 4 °C until analysis for siderophores.

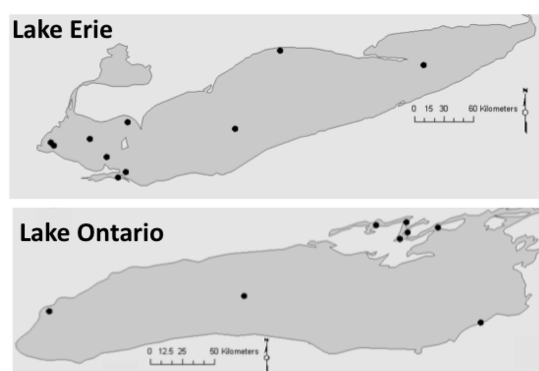

Fig. 1. Location of the sampling sites (•) for siderophores on Lake Erie (upper: Environment Canada stations 222, 452, 478, 879, 880, 882, 885, 966, 970, 973, and 1163) and Lake Ontario (lower: Environment Canada stations 71, 90, 744, 750, 826, 1193, 1194, and 1196) during the summers of 2012, 2013, and 2014.

One-liter samples (1 m depth) of unfiltered water were collected concurrently with each SPE sample and frozen on board the ship. Once back in the laboratory, these samples were thawed and lyophilized to dryness. The contents were transferred to a smaller flask using 50 mL Nanopure distilled water and re-lyophilized to dryness. The contents were reconstituted in 3 mL distilled water, filtered through a 0.45 µm nylon membrane syringe filter, and stored at 4 °C until analysis for siderophores.

Cyanobacterial abundance and chlorophyll-a was estimated from on-board measures of

fluorescence spectra using an Algal Online Analyser (AOA, bbe moledanke, Germany). Net tows (20 µm mesh) were also examined visually using an inverted light microscope at 100-200x. Sample extracts were analysed for hydroxamate siderophores using two methods. High performance liquid chromatography with mass selective spectroscopy (HPLC-MS) or HPLC-PDA was performed using a Waters Acquity TQD tandem mass spectrometer coupled to a Waters Alliance 2895 Separation Module and 2996 PDA detector, using an ACE 5 C18 column (150 x 4.6 mm, 5 µm) fitted with a 2.0 µm prefilter. The instrument detection limit was 9.92 x 10^{-2} nM ferrated DFB by PDA at 425 nm and 2.03 x 10^{-1} nM by mass spectrometry using the TIC channel. The solvent conditions for the HPLC are given in Table 1.

Table 1: HPLC solvent and gradient used for the separation of siderophores.

Time (min)	Flow (mL/min)	0.1% Formic Acid in Water (%)	0.1% Formic Acid in Acetonitrile (%)
0	0.5	95	5
15	0.5	60	40
16	0.5	0	100
26	0.5	0	100
27	0.5	95	5
42	0.5	95	5

Csáky tests were also performed on all samples using a micro-modification of the method described by Gillam *et al.* (1981). All sample volumes were decreased by a factor of 10, samples were hydrolysed in a hot block at 110 °C for 14-16 hr in 13 x 100 test tubes, and absorbance at 543 nm was measured in a Milton Roy Spectronic 3000 Diode Array Spectrometer. Previous work (Morse 1994) has shown that this micromodification gives similar sensitivity to the test described in Gillam *et al.* (1981) with detection limit for a 20 L sample concentrated to a 3 ml final volume of 0.075 µg hydroxamate N/L.

Results and Discussion

The field and laboratory tests indicated the SPE protocol was an effective method to concentrate hydroxamate siderophores from freshwater environments. Solid phase extraction cartridges containing different functional groups gave different recoveries of DFB. SPEs with C18 functionality gave an inconsistent recovery of DFB for a given application volume (Figure 2). Application volumes greater than 10 L led to a recovery of applied DFB below 20%. In contrast, polymeric SPEs consistently gave higher recoveries of DFB than C18 SPEs, maintaining a recovery of >60% at application volumes greater than 10 L.

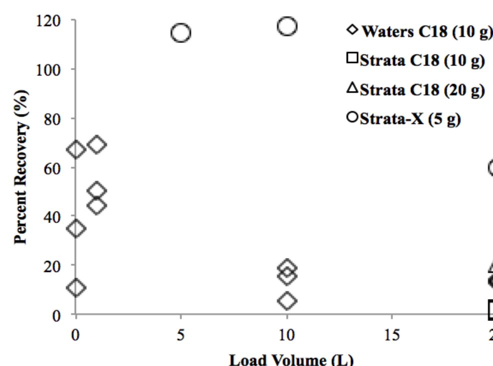

Fig. 2. The percent recovery of DFB from several different solid phase extraction cartridges applied in different application (load) volumes.

A stepwise analysis of the losses indicated the recovery for all steps following elution of the SPE was 100%. Any losses observed were due to differences in the adsorption of the siderophores to the SPE and its subsequent elution with methanol. Analysis of SPE and whole water samples collected from the offshore waters of Lake Erie, and the offshore and inshore Bay of Quinte in Lake Ontario consistently gave negative (non-detectable) results using the Csáky test and by HPLC-PDA-MS. Chlorophyll-a concentrations in these sites ranged from 0.1 µg/L (eastern Lake Erie) to >20 µg/L in the western basin of Lake Erie. A single positive result using the Csáky test was obtained on a SPE eluent from a sample collected from Sandusky Bay in August of 2012. This sample contained 0.28 µg hydroxamate N/L, equivalent to 3.74 µg/L DFB. We were unable to identify which siderophores was responsible for this positive Csáky test using HPLC-PDA and the characteristic ferric-hydroxamate absorbance at 420-440 nm, or by LC-MS using the molecular ion of any of the common hydroxamate siderophores (Winkelmann 1991). This single positive result was obtained from a sample site that frequently shows high levels of cyanobacteria during the summer months (> 20 µg chl-*a*/L in 2012). Microscopic examination of the 2012 sample showed a high abundance of the genus *Dolichospermum* (syn *Anabaena*) in comparison to other cyanobacteria present at the time (*Microcystis, Aphanizomenon,* and *Planktothrix*). The site is located in the channel (11 m) of a relatively shallow bay (average depth 3 m). Further work in needed to

determine if this positive Csáky test corresponds to a potential siderophore produced by the *Dolichospermum* species present. Schizokinen, a siderophore known to be produced by some species of *Dolichospermum* (Goldman *et al*. 1983; Morse 1994) was not observed in the HPLC-PDA traces. Further characterization using LC-MS/MS is in progress. The general absence of hydroxamate-N at most sample locations does not, however, mean that siderophore production is not an important mechanism for iron uptake by Great Lakes plankton communities. The western basin of Lake Erie is relatively shallow and prone to the re-suspension of sediments, and sample material collected from this basin may not have been iron limited. Furthermore, offshore waters in the central and eastern basins generally showed very low levels of both total chlorophyll-a and cyanobacterial-specific pigments. Cyanobacteria in these nutrient-poor waters often showed the presence of heterocysts, suggesting that N-fixation was an important process by which they obtained needed nitrogen. Siderophore formation and N-fixation would compete for available cellular energy and may be mutually exclusive processes in those waters (Hutchins *et al*. 1991). Cyanobacteria present may have produced siderophores with a different functional group than a hydroxamate, for example catechol siderophores are produced by some *Synechococccus* species (Wilhelm and Trick 1994). The SPE extraction protocol and samples collected as part of this work will need to be evaluated for the presence of non-hydroxamate siderophores. Future work to determine the importance of catechol siderophore production on bloom formation in these environments is in progress.

Acknowledgements

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Marine HAB Biology and Ecology

Winter distributions of *Dinophysis* populations: do they help predict the onset of the bloom?

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Abstract

Blooms of diarrhetic shellfish toxin (DST) producers of the genus *Dinophysis* (*D. acuminata, D. acuta*) pose the main threat to the sustainable exploitation of cultivated mussels and other bivalves on the Atlantic coasts of Europe. *Dinophysis* species do not rely on cysts as a seeding strategy. Detection and evaluation of holoplanktonic populations surviving after bloom decline may be the key to predict the initiation of next year's bloom. Three cruises were carried out on the NW Iberian shelf in February 2013 (DINVER 2013), January 2006 (DINVER 2006) and May-June 1993 (MORENA 93) to explore winter (*D. acuminata*) and pre-bloom (*D. acuta*) distributions of harmful microalgal species. Sampling protocols were adapted to be able to detect extremely low densities (1-5 cells l^{-1}) of *Dinophysis* species. Potential inoculum populations in retention areas, as previously described for other species in upwelling regions, were not found on these cruises. Here we explore retrospectively data from these cruises, identify hydrodynamic patterns, and accompanying microplanktonic communities, in an attempt to untangle a crucial question in *Dinophysis* population dynamics: how to predict the initiation of the *Dinophysis* growth season.

Keywords: Dinophysis distribution, overwintering cells, *Dinophysis acuminata*, *Dinophysis acuta*

Introduction

Dinoflagellate species of *Dinophysis* produce lipophilic shellfish toxins (DSP toxins and pectenotoxins) and pose a worldwide threat to sustainable exploitation of shellfish resources (Reguera *et al.* 2014). Endemic blooms of *Dinophysis acuminata* and *D. acuta* cause lengthy shellfish harvesting closures in aquaculture sites on the European Atlantic coast. In the Galician Rías (NW Iberia), *D. acuminata* can be associated with DSP outbreaks within the whole upwelling season (March-October), but *D. acuta* is very seasonal and outbreaks caused by this species usually occur during the autumn upwelling transition (Escalera *et al*. 2006, 2010). Considerable knowledge has been gained on the population dynamics of *Dinophysis* species, but the causes of their interannual variability and the origin of the inoculum remain poorly understood (Reguera *et al*. 2012). In sexual cyst-forming species with mandatory resting periods, yearly recruitment of new cysts to the top sediment layer can be tracked and used in prediction models (Anderson *et al*. 2014). *Dinophysis* species have complex polymorphic life cycles including sexual processes, but the existence of sexual cysts has not been proven either in field populations or in laboratory cultures (Escalera and Reguera 2008). An alternative procedure is to explore the relationship between overwintering mobile cells acting as "pelagic seed banks" (Smayda 2002; Smayda and Trainer 2010) and the initiation of the species growth season. Weekly monitoring has proven to be insufficient to detect rapid changes in numbers due to wind-direction reversals and subsequent DSP outbreaks (Whyte *et al.* 2014). This stresses the need for modeling/operational oceanography approaches.

One objective of the EU project ASIMUTH was the "*Identification of key past events which will be re-analysed and used for training the modeling system*" (www.asimuth.eu). In this framework, we revisited results from three mesoscale cruises carried out on the NW Iberian shelf. Our main question was whether pre-bloom/overwintering distributions of *Dinophysis* species on the shelf are useful to predict the initiation of *Dinophysis* growth season in the Galician Rias Bajas.

Material and Methods

CTD casts and water samples (Niskin bottles) for phytoplankton analyses (Utermöhl method, specimens from the whole chamber at 100X) were collected on three cruises on the NW Iberian shelf. Ekman transport was estimated from model data of the US Navy's Fleet Numerical Meteorology and Oceanography Centre (FNMOC) derived from sea level pressure on a grid of approximately 1°x1° centred at 43°N 11°W, a representative location for the study area.

MORENA 93 (May 5-31, 1993): carried out on board RV *Cornide de Saavedra*, sampled 13 transects (92 stations) perpendicular to the coast between Cape Finisterre (43ºN) and Mondego River (40ºN) at a time of the year when *D. acuta* (target species) is usually below detection levels in the Galician Rías Bajas. Lugol-fixed water samples (250 ml), collected at several depths, were left to settle in glass measuring cylinders over 2d before siphoning out to a final volume of 50ml, sedimentation and counting (detection level, 4 cells l^{-1}).

DINVER 2006 (Jan 31 - Feb 2, 2006) was carried out on the Galician shelf and outer reaches of the Rías Bajas on board RV *Mytilus* in early winter, when *D. acuminata* cells are hardly detectable in the Rías. Stations (47) were chosen after real time simulations of water velocities with the MOHID model (Carracedo *et al*. 2006) so as to include 2 transects and different points located within anticyclonic eddies suspected to act as retention areas for HAB species. Water samples (2.5 l) were passed through a PVC cylinder with a 20-µm sieve, to a final volume (to be measured) of around 50 ml, and 25 ml were sedimented for cell counts (detection level ~1 cell l^{-1}).

DINVER 2013 (Feb 27 - March 1, 2013), on board RV *Ramón Margalef* in mid winter, surveyed 7 transects (45 stations) distributed in a fixed grid. Samples (1-2.5 l) were filtered through nytex filters (20-µm, 47 mm Ø) that were resuspended in 50 ml of filtered seawater with Lugol's solution before sedimentation of 10 ml for counting (detection level, 2-5 cells l^{-1}).

Results and Discussion

<u>MORENA 93</u> started under upwelling conditions followed by relaxation until mid May. From then, until after the cruise, strong and highly variable SW winds caused prolonged downwelling conditions, most unusual at this time of the year (Fig. 1A), and surfacing of the Iberian Poleward Current (IPC, S>35.9). The latter formed a strong tongue-shaped density gradient close to the Portuguese coast (40-42ºN), but nearer to the shelf break off the Galician Rías (42-43ºN) (Fig. 1B).

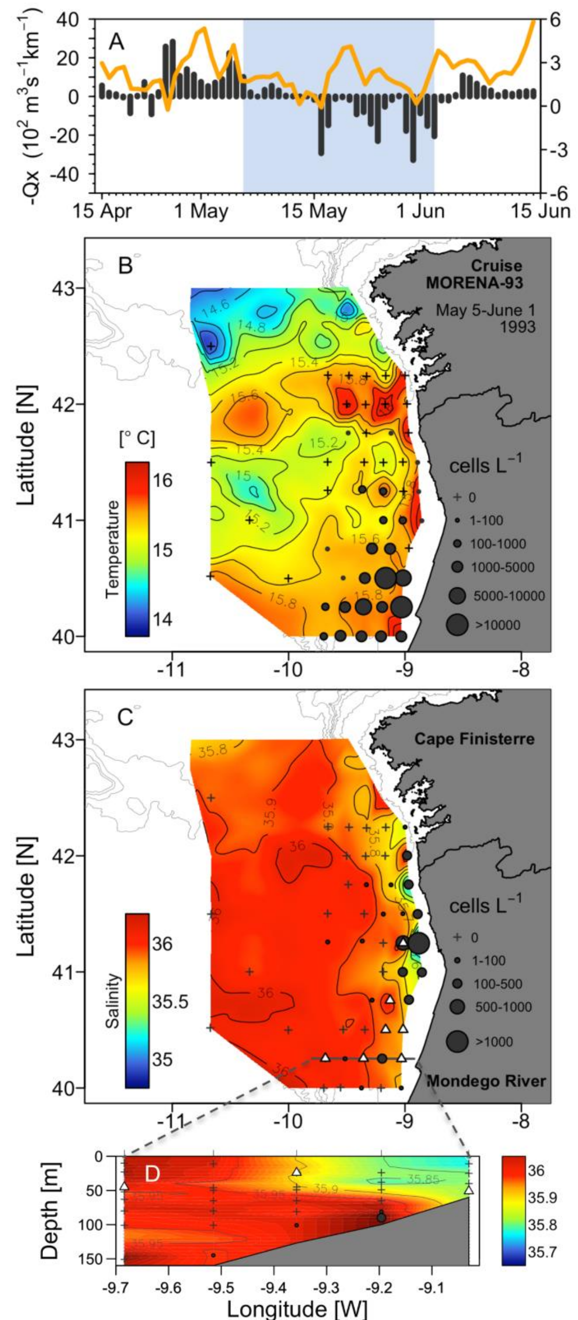

Fig. 1. MORENA 93. A: Daily Ekman transport (orange line shows the 1985-2013 mean; shaded area, the cruise period; B: Temperature (5m) and *G. catenatum* cell maxima; C: Salinity (5m) and *D. acuminata* cell maxima distribution (white triangles represent *D. acuta* ≤8 cell·l^{-1}) and D: Vertical distribution of salinity, *D. acuminata* cells and *D. acuta* presence.

There was a strong latitudinal heterogeneity in the micro-phytoplankton distribution (data not shown) with two well differentiated parts: a northern half with dominance of diatoms and a southern half, dominated by dinoflagellates, including a bloom of the PSP agent *Gymnodinium catenatum*, with cell maxima in the pycnocline at about 50m (Fig 1B). Moderate to low densities of *D. acuminata*, which had been reported by the Galician monitoring programme since February, were found near the coast in the whole area. *D. acuta* at extremely low densities (4-8 cell l^{-1}) was detected at 7 stations in the southern half. A vertical section showed that these cells were in the top 50-m layer, whereas *D. acuminata* cells were near the seabed (Fig. 1D).

DINVER 2006. The objective of this cruise was to look for overwintering populations of *D. acuminata*. Conditions in the outer reaches of the Galician Rías showed temperature inversions, typical for the area in mid winter; the upwelling season had not started. Micro-phytoplankton was very scarce, but with a good contribution of large dinoflagellates (*Ceratium azoricum*, *C. candelabrum*, *C. pentagonum)* that occur this time of the year associated with the Iberian Poleward Current. In addition, there were scattered cells of *G. catenatum* and *D. acuta* that were remains of intense blooms of these species in November 2005 (Pizarro *et al*. 2008) that caused harvesting closures due to DSP (until the end of January) and PSP (until May), toxins above regulatory levels until mid-spring of the next year (ICES 2006). A few isolated cells of *D. acuminata* were detected at only 2 stations in the outer reaches of the northern margins of Ría de Muros and Ria de Pontevedra (Fig. 2).

DINVER 2013: The upwelling season had started, and large diatoms (e.g. *Ditylum brightwellii*) and *Chaetoceros* spp. were dominant in the photic zone throughout the Galician shelf. *D. acuminata*, which had been detected in the Rías Baixas all through the winter, was found in low numbers (< 40 cell l^{-1}) everywhere except at some stations in the northernmost transects. Densities were slightly higher in the mouths of Ría de Vigo and Ría de Pontevedra due to advection.

The three cruises were carried out coinciding with anomalous conditions for their time of the year, but we examined retrospectively the information we can draw from each one concerning prediction of the initiation of forthcoming blooms of *Dinophysis* species.

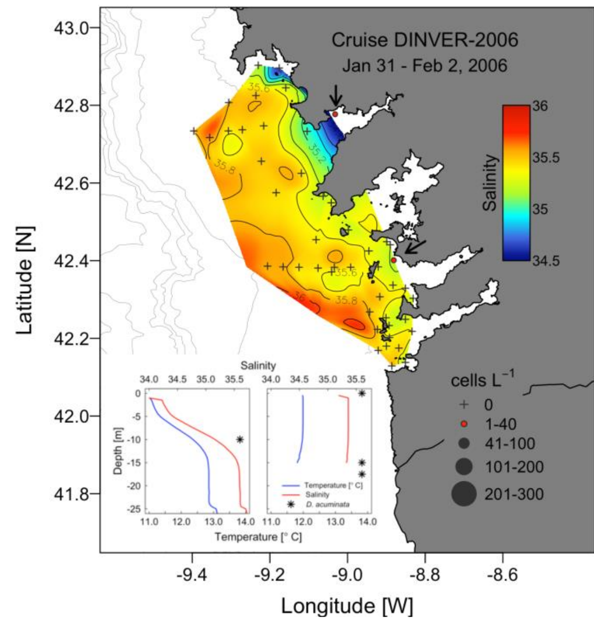

Fig. 2. Surface salinity distribution during DINVER 2006 cruise. Vertical profiles correspond to the 2 stations (red dots and black arrows) where scattered cells of *D. acuminata* were found.

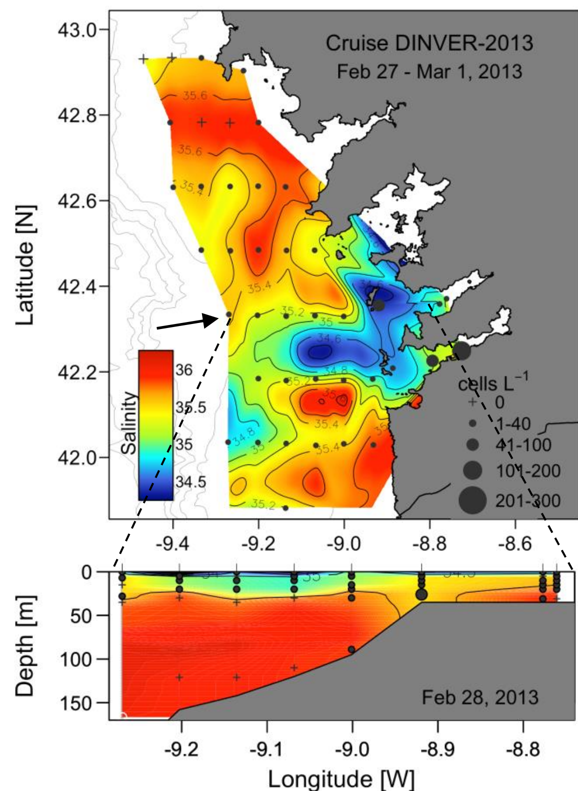

Fig. 3. Salinity (5m) and *D. acuminata* distribution during DINVER-13, and a vertical profile of a transect from Ría de Pontevedra to the shelf (marked with an arrow).

In the case of MORENA 93, conditions in May recalled those typical in September-October when the upwelling season is finished. But still *D.*

acuta, a species whose autumn blooms in the Galician Rías usually precede (a few days difference) those of *G. catenatum*, was detected only in the southern half of the survey area. This observation confirms this species is a seasonal (late summer-autumn) visitor to the Galician Rías. Later data have shown blooms of this species have their epicentre in Aveiro (40.7ºN), Portugal, and information on *D. acuta* developments there constitute the most reliable early warning for later blooms to develop in Galicia (Moita *et al*. 2005; Escalera *et al*. 2010).

DINVER 2006 followed exceptionally late (November) and intense blooms of *D. acuta* and *G. catenatum* the previous year, and the scattered cells of these species detected did not serve our objectives. No overwintering or pre-bloom cells of *D. acuminata* were found in potential retention areas, i.e. anticyclonic eddies identified by the MOHID model predictions in real time, in contrast with findings of "HAB incubators"(of *D. acuminata* in the Bay of Biscay, see Xie *et al.* 2007, and of *Pseudo-nitzschia* spp. in the Juan de Fuca Eddy, NW USA, see Trainer et al. 2009) associated with this kind of hydrodynamic feature. Nevertheless, 2006 was also peculiar concerning its very late initiation of the *D. acuminata* growth season, in late June, following the occurrence of an exceptional bloom of *D. ovum* (Pizarro et al., 2013). The lesson from this cruise is that the absence of winter populations of *D. acuminata* in the Rías Bajas and shelf in early February is a good sign for not expecting early (before June) DSP closures caused by this species. This conclusion was later confirmed in a time series analysis for identification of the causes of phenological changes in the onset of *D. acuminata* blooms (Díaz *et al.* 2013).

DINVER 2013 was carried out four weeks later than DINVER 2006, looking for a time window closer to the initiation of the *D. acuminata* growth season. But 2013 was "different" in having a very early initiation of the upwelling season (favourable for the onset of *D. acuminata* growth, see Díaz *et al*. 2013) and cells of these species were present in the water column throughout the winter. In fact, shellfish harvesting closures due to DSP toxins in the Rias Bajas started in early April. The lesson from this cruise is that detection of *D. acuminata* cells in the Galician Rías and shelf through the winter poses a higher risk of early onset of DSP outbreaks.

Acknowledgements

We thank the Galician Monitoring Programme (www.intecmar.org) for weekly reports on HABs and environmental conditions. Funded by EU project ASIMUTH (EC FP7-SPACE-2010-1 grant agreement number 261860) and Spanish (MINECO, Programa RETOS) project DINOMA (CGL2013-48861-R). Patricio A. Díaz had a PhD student fellowship from BECAS–CHILE, CONICYT.

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Testing alternative prey species for *Mesodinium rubrum* and *Dinophysis acuta*

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Abstract

The *Dinophysis acuta* is a mixotrophic dinoflagellate that requires organelles from other microorganisms in order to thrive. Its culture in laboratory conditions relies on the presence of *Mesodinium rubrum*, a ciliate prey species that feeds on *Teleaulax* sp., a cryptophyte that provides the organelles transmitted through this food chain. Recent literature has suggested that wild samples of *Dinophysis* contain organelles from other genera, hence we set up a series of tests to explore other candidates for this food chain. Small-scale screening tests, performed in 24-well plates and lasting 2 weeks, were used to identify suitable alternative preys among microalgae strains from the Cawthron Institute Culture Collection of Micro-algae (CICCM). *Chrysochromulina hirta*, *Chrysochromulina simplex*, *Cryptomonas* sp., *Gymnodinium simplex*, *Heterocapsa triquetra, Pavlomulina* sp. and *Teleaulax* sp. (as control) were tested for ingestion by *Mesodinium rubrum*. Additionally, *Akashiwo sanguinea*, *Alexandrium minutum*, *Amphidinium massartii*, *Gymnodinium simplex* and *Pavlomulina* sp. were tested for direct ingestion by *Dinophysis acuta*.
A second test, in 70mL containers and lasting 1.5 months, was performed with prey species selected from the screening test for *Mesodinium rubrum*. The results indicate *Pavlomulina* sp. and *Chrysochromulina simplex* as promising preys for *Mesodinium rubrum*. Mucus traps and pigmented *Dinophysis acuta* cells (i.e. with organelles) were observed in the wells containing *Pavlomulina* sp., suggesting an active intake but not enough to sustain *Dinophysis acuta* culture growth.

Keywords: Dinophysis acuta, *Mesodinium rubrum*, mixotrophy, mucus trap

Introduction

In 2006, Park et al. published the successful culture of *Dinophysis acuminata* based on the delivery of a ciliate prey, *Mesodinium rubrum*, which in turn was fed with *Teleaulax* sp. (Cryptophyceae). This seminal work set the basis for the culture of several *Dinophysis* species, relying on this food chain, where organelles of the photosynthetic *Teleaulax* are used by *Mesodinium* and finally by *Dinophysis* (reviewed by Hansen *et al.* 2013). Nevertheless, some data published by Nishitani *et al*. (2012) from wild *Dinophysis mitra* cells suggest that its organelles do not have a solely Cryptophyceae origin, but they can be from Haptophyceae (specially *Chrysochromulina* species), Prasinophyceae, Dinophyceae, Pelagophyceae, Bolidophyceae, Bacillariophyceae and other unidentified origins. We performed experiments investigating potential prey species of this consortium and on *Dinophysis* feeding behaviour.

Material and Methods

Cell isolation

Mesodinium rubrum cells were isolated from Wedge Point (Queen Charlotte Sound, Marlborough, New Zealand) in June 2012, and maintained in 12-well tissue culture plates (TCP) containing 0.22 µm filtered F/2 medium without silica (Guillard and Ryther 1962) at a constant temperature of 18 +/- 1ºC and light conditions of 12 h light : 12 h dark, 70-100 $\mu mol.m^{-2}.s^{-1}$ cool-white fluorescent light (hereafter referred to as standard conditions). *Teleaulax amphioxeia*, supplied by Dr. S. Nagai (NRIFS, Japan, see acknowledgements) was provided as prey.
Dinophysis acuta cells were isolated from Takaka (Golden Bay, New Zealand) in May 2013, and maintained in 12-well TCP containing media under standard conditions. *Mesodinium rubrum* was delivered as prey.

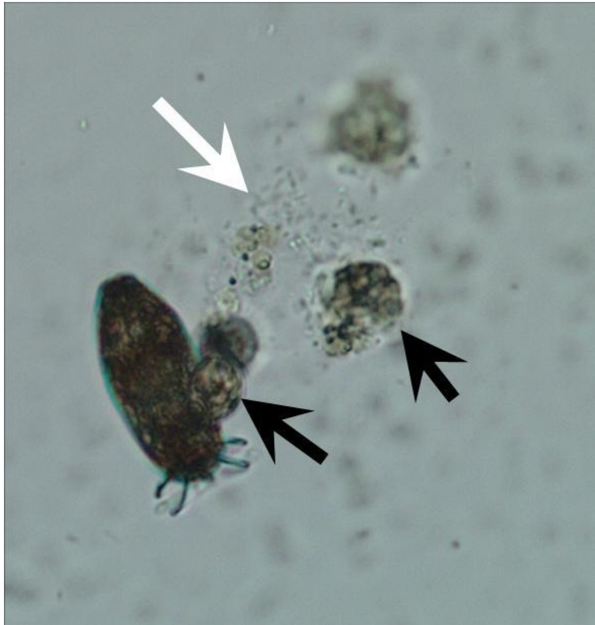

Fig. 1. A live *Dinophysis acuta* cell (motile and pigmented on left) and extracellular mucus that may act as a toxic trap (white arrow) with *Mesodinium rubrum* embedded (black arrow).

First trophic level experiment 1
Single cells of *M. rubrum* were isolated in each well of a 24-well TCP containing 1 mL of media. The following candidate prey species were added; one prey type per plate: *Teleaulax amphioxeia*, *Cryptomonas* sp. (CAWCr01), *Chrysochromulina simplex* (CAWP20), *Gymnodinium simplex* (CAWD86), *Chrysochromulina hirta* (CAWP02) and *Pavlomulina* sp. (CAWP21). Negative controls were *M. rubrum* without any prey addition. The plates were kept under standard conditions and checked daily for two weeks in order to determine *M. rubrum* survival and growth.

First trophic level experiment 2
Chrysochromulina simplex (CAWP20) and *Pavlomulina* sp. (CAWP21) were selected based on results from the first trophic level experiment. *Teleaulax amphioxeia* was the positive control with no addition of prey species as the negative control; resulting in a total of 4 experimental groups. *Mesodinium rubrum* culture was upscaled and a set of 50 mL subcultures were started in 70 mL sterile polystyrene containers Three replicates were used for each experimental group; 12 containers in total. The containers were kept under standard conditions and sampled weekly in order to estimate *M. rubrum* densities.

Second trophic level experiment
Five to ten cells of *D. acuta* were placed in 12-well TCP containing 3 mL of media. The following candidate prey species were added; one prey type per well: *Akashiwo sanguinea* (CAWD01), *Alexandrium minutum* (CAWD11), *Amphidinium massartii* (CAWD156), *G. simplex* (CAWD86) and *P. kotuku* (CAWP21). The plates were kept under standard conditions and checked daily in order to detect the mucus trap mechanism used for hunting *M. Rubrum*, and *D. acuta* cells vitality and culture growth (Figure 1).

Results and Discussion

First trophic level experiment 1
The survival of *M. rubrum* cells was not consistent among wells or containers provided with the same potential prey. Few *M. rubrum* cells of the 24 isolated per plate were able to divide, although the maximum cells per well and the day at which this maximum was achieved differed between groups (Table 1).

Table 1. First trophic level experiment 1 results. The prey species selected for First trophic level experiment 2 are underlined.

	Maximum *M. rubrum*	
Preys added	Cells / well	Day
No preys added	28	12
Teleaulax amphioxeia	75	>15
Cryptomonas sp.	4	12
Chrysochromulina simplex	26	16
Gymnodinium simplex	4	13
Chrysochromulina hirta	29	12
Pavlomulina sp.	59	9

First trophic level experiment 2
Population dynamics of each organism (*M. rubrum* and potential prey) were also different among containers with the same combinations of *M. rubrum* / potential prey. In some cases the potential prey population outnumbered *M. rubrum* population until its collapse, maybe due to variations in the culture environment. The surviving cultures showed differences in the maximum *M. rubrum* cells densities and the culture dynamics (Figure 2).

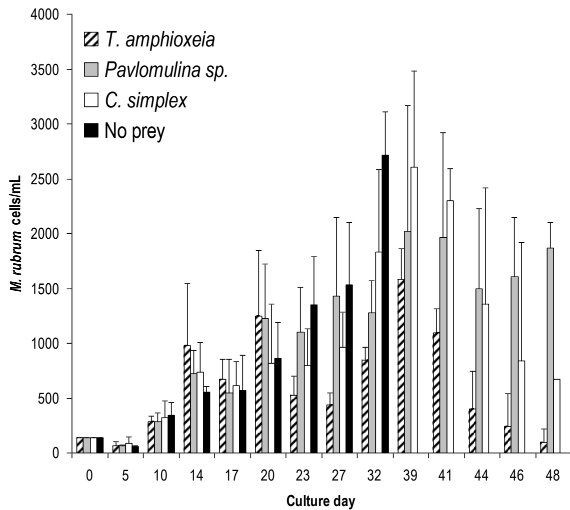

Fig. 2. First trophic level experiment 2 results. Note that the experimental group "no preys added" finished on day 32. One container of *Pavlomulina* sp. group was discarded on day 39, one container of *C. simplex* group was discarded on days 44 and 48, and one container of *T. amphioxeia* group was discarded on day 46.

Second trophic level experiment

Deformed trapped but living cells of *A. minutum* (Figure 3) and *Pavlomulina* sp. (Figure 4) were observed embedded in the mucus. *Dinophysis acuta* cells were lightly pigmented but did not divide and eventually died in the wells containing *A. minutum*. In the wells containing *Pavlomulina* sp. , some *D. acuta* (Figure 4) cells were highly pigmented and were able to divide once or twice, but eventually all the cells also died. In the wells provided with *A. sanguinea*, *A. massartii* or *G. simplex*, *D. acuta* cells did not present any signs of vitality, mucus trap was not detected, and *D. acuta* cells eventually died.

Conclusions

Cell-specific differences in *M. rubrum* and *D. acuta* that affect the outcomes of the experiments were observed. *Mesodinium rubrum* cultures can grow on prey species other than *T. amphioxeia*, although the bloom maxima seems to be later and reach lower densities than the *T. amphioxeia* cell maxima. *Dinophysis acuta* cultures could be maintained, but cultures do not grow when being fed with *Pavlomulina* sp.; the other tested potential dinoflagellate preys, have not been able to keep *D. acuta* alive.

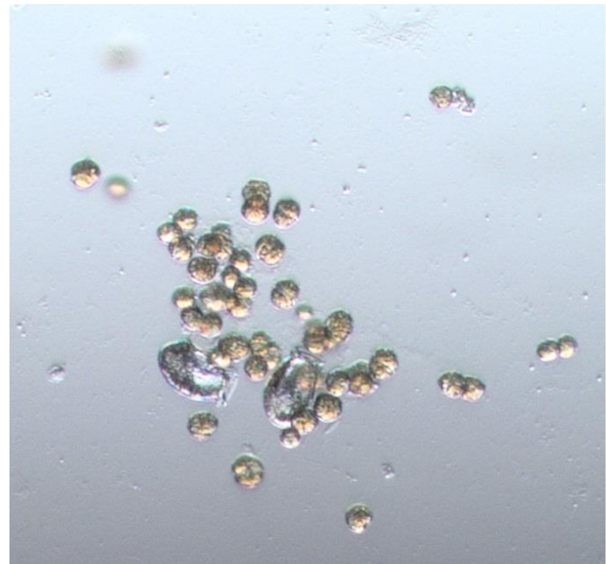

Fig. 3. *Alexandrium minutum* trapped in the mucus, some already deformed and *D. acuta* cells lightly pigmented.

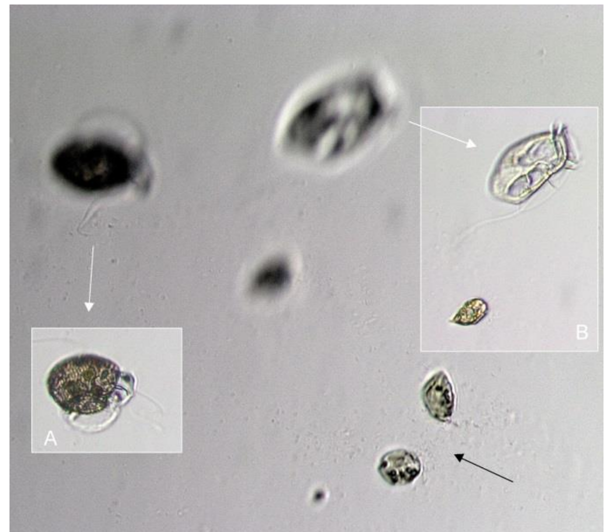

Fig. 4. Mucus trap with deformed *Pavlomulina* sp. cells trapped (black arrow); motile *D. acuta* cells both pigmented (focused image A) and not pigmented (focused image B).

The work presented here exploring an alternative prey consortium for cultured *Dinophysis* genera shows results similar to other published works, where *D. acuta* cells can be maintained in the presence of other prey genera but appear to flourish only in the presence of *Mesodinium* sp**.** This study has not been exhaustive of all possible prey genera and when hence future work should still be carried out to continue searching for alternative prey consortia.

Acknowledgements

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Role of organic nutrients originated from *Mesodinium rubrum* and *Teleaulax amphioxeia* cultures in the growth and toxin production of *Dinophysis acuminata*

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Abstract

As a causative species of diarrhetic shellfish poisoning (DSP), *Dinophysis acuminata* is widely studied on its physiological and toxicological characteristics by alternating the factors including temperature, illumination, prey quantity and dissolved inorganic nutrient. However, the factor of organic nutrients has not been well studied. Therefore, we conducted a series of experiments to investigate how the organic material originating from its prey, *Mesodinium rubrum* and *Teleaulax amphioxeia*, influence the growth and toxin production of *Dinophysis*. The results showed that *Dinophysis* had significant growth when fed with an extract from *M. rubrum* culture, with the growth rate of 0.12 d^{-1}. The highest growth rate (0.25 d^{-1}) and highest cell density (3902±234 cell mL^{-1}) of *D. acuminata* were detected when supplied with half ciliate debris and half intact ciliate cells, indicating that the uptake of organic substrate from *M. rubrum* cultures would possibly be enhanced by the present of living prey. However, no growth was found when supplied with either intact *Teleaulax amphioxeia* cells or its debris. Cellular DTX1 and/or OA were significantly greater in the cultures containing the cell debris and organic substrates from the *M. rubrum* culture compared to the control, suggesting that dissolved organic substances may play a role in enhancing the toxin production. The increase in the number of *D. acuminata* cells led to elevated total toxin concentration (intra + extracellular toxins, ng/mL of culture) of OA and DTX1. Our results suggest that organic nutrients from *M. rubrum* can be utilized by *Dinophysis* as a nutrient source that influences toxin production.

Keywords: Dinophysis acuminata; *Mesodinium rubrum*; *Teleaulax amphioxeia*; organic nutrient; diarrhetic shellfish poisoning (DSP); Okadaic acid (OA); dinophysistoxin (DTXs)

Introduction

Dinophysis acuminata is a cosmopolitan species that causes Diarrhetic Shellfish Poisoning (DSP) events worldwide (Reguera *et al*. 2012). As an obligate mixotrophic species, *Dinophysis* requires a unique three - stage food chain, cryptophyte (photosynthetic nanoflagellate) - *Mesodinium rubrum* (phototrophic ciliate) - *Dinophysis* to maintain its growth (Park *et al.* 2006). Autecology studies and microscopic observations have revealed that *Dinophysis* use a peduncle to feed (Park *et al.* 2006, Tong *et al*. 2014) and food vacuoles (Jacobson & Andersen 1994) contain of *M. rubrum* plastids and other organelles (e.g. mitochondria and lipids). Interestingly, the stolen cryptophyte chloroplasts (kleptoplastids) are maintained in both *Mesodinium* and *Dinophysis* cells during this complex feeding process, providing a direct linkage between prey and predator (reviewed in Gagat *et al*. 2014) .

The effects of environmental factors (temperature, illumination, dissolved inorganic nutrients and food availability) on the growth and toxin production of *Dinophysis* have been widely investigated (Kamiyama *et al*. 2010, Nagai *et al*. 2011, Tong *et al*. 2011, Nielsen *et al.* 2013, Tong *et al*. in press). Intracellular and extracellular toxin components and proportions at different growth phases have also been investigated (Nagai *et al*. 2011, Tong *et al*. 2011). However, the food availability (prey status, prey type and organic substances or particles) have received less attention (Nagai *et al.* 2011). Here we investigated the possibility of *D. acuminata* utilizing organic extraction originated from its prey, *M. rubrum* and *T. amphioxeia*, and the effects on the growth and toxin production of *Dinophysis*.

A. Lincoln MacKenzie [Ed]. *Marine and Freshwater Harmful Algae 2014*. Proceedings of the 16th International Conference on Harmful. Algae. Cawthron Institute, Nelson, New Zealand and the International Society for the Study of Harmful Algae (ISSHA)

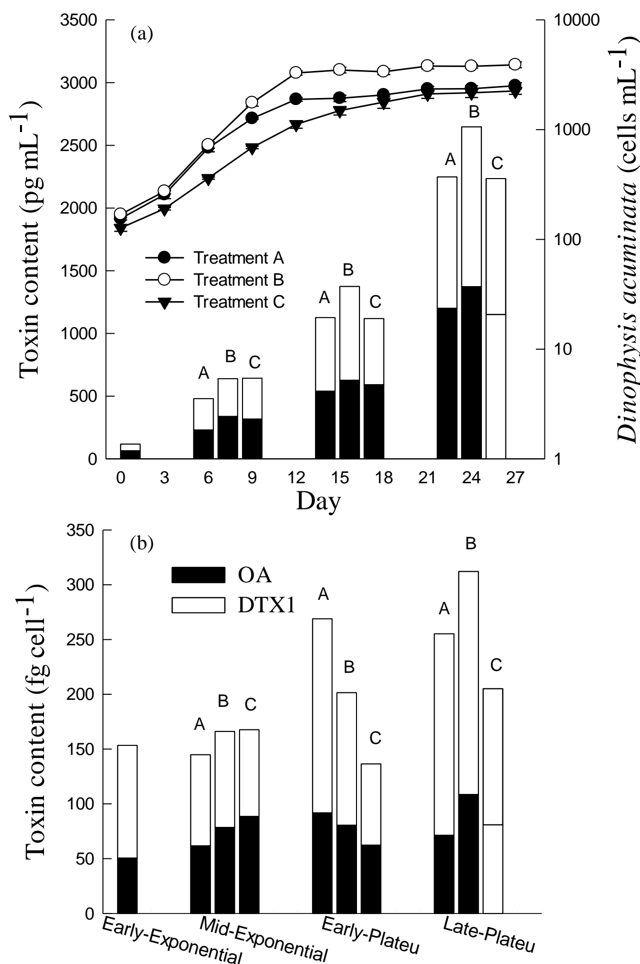

Fig 1. *D. acuminata* growth and total toxin content (a) and cellular toxin partitioning (b) of treatments A, B & C. .

Material and Methods

Cultures. An uni-algal culture of *D. acuminata* (DAEP01) was previously isolated from Eel Pond, Woods Hole, MA in September of 2006 (Tong *et* al. 2011). The ciliate *M. rubrum* and cryptophyte *T. amphioxeia* were isolated from Inokushi Bay in Oita Prefecture, Japan, in February of 2007 as described in (Nishitani *et al*. 2008). All cultures were maintained in modified f/6 medium, which was prepared with 1/3 nitrate, 1/3 phosphate, 1/3 metals, and 1/3 vitamins of f/2-Si medium. Cultures were maintained at 15°C with dim light on a 14h light: 10h dark photocycle.

Growth experiment. A series of experiments were conducted to investigate the physiological and toxigenic characteristics of *D. acuminata* (Table 1). Initial concentration of *D. acuminata* was 150 cell mL^{-1} in all treatments (A-G). Treatment G was set as control. For each treatment, triplicate, 1L flasks with 500 mL of f/6-Si medium were inoculated. Cell count samples of 1.2 mL were taken every three days, fixed by 3% (v/v) formalin solution and enumerated microscopically using Sedgewick-Rafter counting chamber at 100X magnification.

Table 1. The response of *D. acuminata* growth to various prey treatments. Initial density of *D. acuminata* in each treatment was 150 cell mL^{-1}. Treatment G was set as control.

Treatment	Prey type	Prey density (cell mL^{-1})
A	*M. rubrum*	3000
B	*M. rubrum*	3000 (50% crushed)
C	*M. rubrum*	1500
D	*M. rubrum*	3000 (100% crushed)
E	*T. amphioxeia*	15000
F	*T. amphioxeia*	15000 (100% crushed)
G	none	—

Toxin analysis. Cells and media of *D. acuminata* cultures were harvested separately and analyzed for toxin at four different growth stages of *D. acuminata* for treatments A~C. For treatments D~G, due to insufficient biomass during the experiments, toxin samples were only collected and analyzed once at late plateau phase. Details of toxin collection and preparation are described in Tong (*in press*). In brief, cells were separated from the medium using a 15-µm Nitex sieve and rinsed into a pre-weighed 15-mL centrifuge tube. Aliquots (200 µL) were pipetted from the mixed sample into separate micro-centrifuge tubes containing 1.0 mL of filtered seawater and 37 µL formalin solution (3% v/v) to later determine the cell concentrations in the harvested cell concentrate (Tong *et al.* 2010). The 15-mL tube was reweighed to determine the volume of harvested *Dinophysis* cells and then frozen at -20 °C. The toxin extraction was described in (Smith *et al*. 2012).

A Waters Ultimate 3000 LC system (Dionex, USA) and AB 4000 triple quadrupole mass spectrometer system (AB SCIEX, USA) coupled with electrospray ionization was used for the toxin analysis. Chromatographic separation was performed using a Waters X-Bridge C18 column (3.0 x 150 mm; 3.5 µm particle size). OA and

DTX1 were analyzed in negative ion mode. Mobile phase: eluent A was water and eluent B was acetonitrile -water (90:10, v/v), both containing ammonium water (0.05%). Toxins were eluted from the column with 90% eluent B at a flow rate of 0.4 mL min^{-1}. The toxin concentration was determined by comparing the peak areas with standard toxins for OA and DTX1, which were purchased from the National Research Council, Canada.

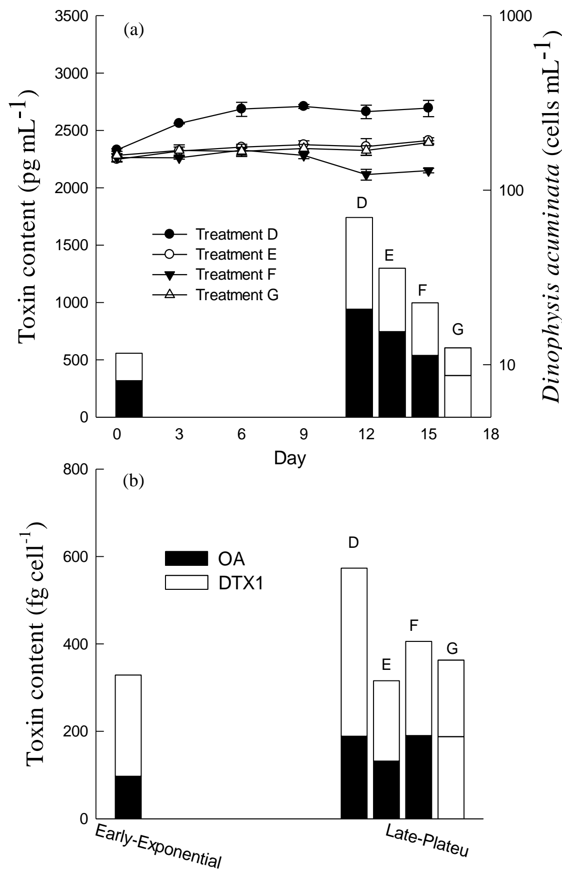

Fig. 2. *D. acuminata* growth and total toxin content (a) and cellular toxin partitioning (b) of treatments D, E, F & G.

Results and Discussion

During 30 days incubation, cell abundance of *D. acuminata* in the four treatments (A~D), containing living *M. rubrum* cells and/or *M. rubrum* debris, increased significantly. The growth rates were 0.23, 0.25, 0.18 and 0.12 d^{-1} in the Treatment A, B, C and D, respectively. No obvious difference of growth rates was observed in Treatment A and B, which contained potential same amount of ciliate prey (Table 1 and Fig. 1 and 2). Even the growth of *Dinophysis* with feeding *M. rubrum* debris (Treatment D) was confirmed, whereas the growth rate and biomass were significantly lower than those in other treatments with living *M. rubrum* cells. The best growth, with the highest concentration of ca.3900 cell mL^{-1}, was found in Treatment B, half *M.rubrum* cells and half debris. No obvious difference in biomass was observed between Treatment A and C, with *D. acuminata* fed on *M. rubrum* of 3000 and 1500 cell mL^{-1}, respectively. In Treatment A, B and C, with living prey, cells of *M. rubrum* increased in the first three days then exponentially decreased until being completely consumed at around 15-18 days (data not shown). Undoubtedly, no growth of *Dinophysis* was observed when fed with living cryptophyte cells and/or debris.

D. acuminata was identified as a Model IIIB mixotroph, which was referred to the species "that can harbor algal endosymbionts or sequester algal plastids but require particulate food for continued growth" (Stoecker 1998). *D. acuminata* was capable of utilizing organic particles (*M.rubrum*) under certain level of light (Park *et al*. 2006, Tong *et al*. 2011, Nielsen *et al.* 2012, Smith *et al*. 2012) while dissolved inorganic nitrate and phosphorus are not assimilated by *D. acuminata* (Tong *et al*. in press). No significant growth of *D. acuminata*, isolated from Harima Nada Japan, was observed when fed with cell debris and dissolved organic substances originating from *M. rubrum* (Nagai *et al.* 2011). In contract, significant growth of our isolate of *D. acuminata*, from Massachusetts, USA, was observed under similar prey condition as previous study (Nagai *et al.* 2011). Ammonium was reported to display a high affinity to *D. acuminata* off the west coast of South Africa (Seeyave *et al*. 2009), indicating the possibility of uptake NH_4^+ by *Dinophysis*. The growth and biomass (Treatment D) were not as high as those fed with living *M.rubrum* cells and cells of living plus debris (Treatment A, B and C). The difference might be induced by the specific character of *Dinophysis*. All these suggested that our *Dinophysis* isolate was capable of utilizing the cell debris and organic substances from *M. rubrum* (Fig. 2). Even more, the uptake of these organic substances might be enhanced when living prey was present (Treatment B, Fig. 1). There were several reasons that might influence the results. 1) *M. rubrum* synchronously benefited from the nutrient-rich medium given that the ciliates could be stimulated by dissolved nutrient (Sagert *et al.* 2005,Tong *et al*. in press) and in turn, contribute to the growth of their predator.

However, no obvious growth of the ciliates in the mixed culture were observed (data not shown). 2) *D. acuminata* could maintain its metabolism and have slow growth when simply supplied ciliate cell debris. The dissolved organic material (DOM) leaks out of intact cells after ultrasonic decomposition of the ciliate cultures. Since the cultures are non-axenic, heterotrophic bacteria might increase in biomass and potentially impact the results. 3) The physiological status of the cells (e.g. growth characters, nutrient status) might affect the utilization of various nutrient substrates (Glibert *et al.* 2004). This leads to another assumption that the plastids in *Dinophysis* ingested from living ciliates could initiate and/or stimulate the ability of *D. acuminata* using organic nutrients in the media. The pore plate of *Dinophysis* is likely to be flexible enough to be pushed aside when "pipetting" organelles using peduncle (Jacobson & Andersen 1994) which makes the cell a temporary open system through the cytostome and pore plate. Organic substrates may be absorbed when *Dinophysis* are physically connected to the ciliate.

Cellular OA and DTX1 contents increased during the growth of *Dinophysis* and reached the maximum contents of 92 and 184, 109 and 204, 89 and 124 fg cell^{-1}, after treatments A, B and C, respectively (Fig.1b). Organic nutrients originated from *M. rubrum* and *T. amphioxeia* might be the driver of OA and DTX1 toxin production by *Dinophysis* (Fig 1b and 2b). The toxin results showed that cellular toxin contents of OA and DTX1 were significantly higher at the late plateau phase at Treatment B (Fig. 1b) and DTX1 at Treatment D (Fig. 2b). Interestingly, cellular OA content, 132 fg cell^{-1}, was significantly lower when fed with an extract of the cryptophyte (Fig. 2b). Similar to our toxin results, cell debris and organic substrates originated from *M. rubrum* were documented to be available for toxigenic process rather than growth of a Japanese *Dinophysis* isolate (Nagai *et al.* 2011), and the toxin content was much lower than *Dinophysis* feeding on living ciliates. The increase in the number of *D. acuminata* cells led to elevated total toxin concentration (intra + extracellular toxins, ng/mL of culture, Fig. 1a and 2a) of OA and DTX1. Simply put, more *Dinophysis* cells in the system, resulted in more total toxin.

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Risk-Monitoring, Modelling and Mitigation (M3-HABs) of benthic microalgal blooms across the Mediterranean regions

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Abstract

The pan-Mediterranean project M3-HABs regarding monitoring of harmful algal blooms, with particular reference to the benthic dinoflagellate *Ostreopsis,* started in 2014 in the framework of the ENPI-CBCMED Programme. The specific objective of the project is to provide a common strategy for monitoring benthic toxic microalgae, through the development of new, more efficient and common procedures and protocols, making the process mostly cost and time effective, allowing for most efficient monitoring designs, increasing the knowledge on environmental drivers affecting *Ostreopsis* blooms and translate this into a forecasting tool, and improving the general awareness of the risks related to *Ostreopsis*. The following results are expected: a larger awareness of the risks associated with the *Ostreopsis* blooms, an appropriate diffusion of cautionary measures set up, the production of common monitoring protocols, the development of new technologies for species-specific identification and counting and the build-up of prediction models in order to prevent and reduce risk factors for the environment, human health and economic activities. The project will improve the establishment of solid networks along Mediterranean coasts to cope with *Ostreopsis* emergencies, providing the target groups common and intercalibrated protocols, in order to have comparable samplings in space and time through the Mediterranean Sea.

Keywords: HABs, *Ostreopsis*, automated counting tool, HABs monitoring, HABs modelling

Introduction

The occurrence of benthic HABs has increased worldwide, both in terms of frequency, magnitude and geographic distribution (Shears and Ross 2009). Proliferations of the toxic benthic dinoflagellates belonging to the *Ostreopsis* genus have so far been reported along European Mediterranean coasts in Italy, Spain, France, Croatia and Greece, and *Ostreopsis* occurrence has been recently reported also in other Mediterranean countries, such as Tunisia, Egypt and Lebanon (Fig.1).

The pan-Mediterranean project M3-HABs has started in January 2014 in the framework of the ENPI-CBCMED Programme and is presently working in order to increase awareness and implement measures to manage benthic harmful algal blooms in the Mediterranean basin through the development of common monitoring procedures, automated counting methods and predictive modeling tools.

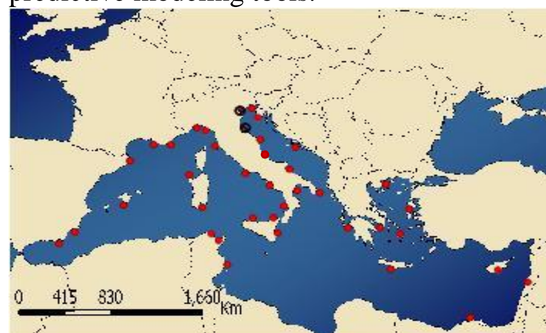

Fig.1. *Ostreopsis* occurrence along Mediterranean coasts.

The M3-HABs project is coordinated by the National Interuniversity Consortium for Marine Sciences (CoNISMa). Project partners are from

Italy (National Research Council-Institute of Biophysics - CNR-IBF, Regional Agency for Environmental protection of Liguria - ARPAL, and two enterprises, OnAir and DHI Italy), from France (Laboratoire d'Océanographie de Villefranche - LOV and Université Nice Sophia Antipolis- UNS), from Tunisia (National Institute of Marine Sciences and Technologies - INSTM) and from Lebanon (National Council for Scientific Research - CNRS). Additionally, 15 associated partners from research institutions, environmental agencies, aquaculture enterprises and media participate in the project.

Communication and Capitalization (WP 2 & 3)
These WPs aim at the visibility and long-term dissemination and exploitation of project results and networking. A project website has been created (http://www.m3-habs.net/) and flyers, brochures and an informative video have been produced. Informative panels, providing information to the larger public about *Ostreopsis* and associated risks, will be deployed along project partners' coastlines during the second year of the project.
Capitalization of achievements reached in the framework of the project will be guaranteed mainly through students' training, through Summer Schools and mobility grants. The first Summer School about "Taxonomy, Phylogeny and Ecology of the *Ostreopsis* genus" took place in June 2014 in Lebanon; the second one, concerning "*Ostreopsis* bloom modelling" will take place in May 2015 in Tunisia. Additionally, the establishment of an *Ostreopsis* network at the Mediterranean level that will survive after the end of the project is expected, involving all project and associated partners but also all interested scientists.
The other four work-packages are more research and technology development focused.

Opto-electronics system and algorithm for cell identification and counting (WP4)
Microalgae identification and counting are currently performed using standard optical microscopy methods to assess phytoplanktonic or phytobenthic specific taxa and their relative abundances. However, the traditional light microscopy identification and quantification methods can be difficult and not cost effective, because they require a great deal of taxonomic expertise to identify species, in addition to being time consuming and impractical for processing a large number of samples. In the framework of M3-HABs project, the development of opto-electronic and molecular tools for automatic *Ostreopsis* cells counting is expected. The optical set-up and acquisition engine to obtain multi-focal images have been produced during the first year of the project, thanks to the collaboration of CNR-IBF, OnAir and CoNISMa. Enhanced images, including a full three dimensional map of the observed samples, have been obtained through Quantitative Phase Microscopy (QPM) reconstruction methods, adding a huge amount of information to be fed to a dedicated cell identification and counting software. The software implemented a detection and statistical classification algorithm, trained to reproduce the performance of a human expert working through the same images, by applying a machine learning approach. *Ostreopsis* spp. cells collected along partners' coastlines have been imaged in order to capture all the morphological variability that each species/morphotype can display. This will allow producing confidence intervals in morphological measurements. The system will be tested in all partners' countries for adapting it to the morphological variability of *Ostreopsis* spp. in the different regions. Furthermore, the application of molecular technological advances in real time quantitative PCR (qPCR) provides additional tools developed in the framework of the project, that allows to identify toxic algae more rapidly and accurately (Casabianca *et al.* 2014), and to confirm morphological species identified through traditional and opto-electronic methods.

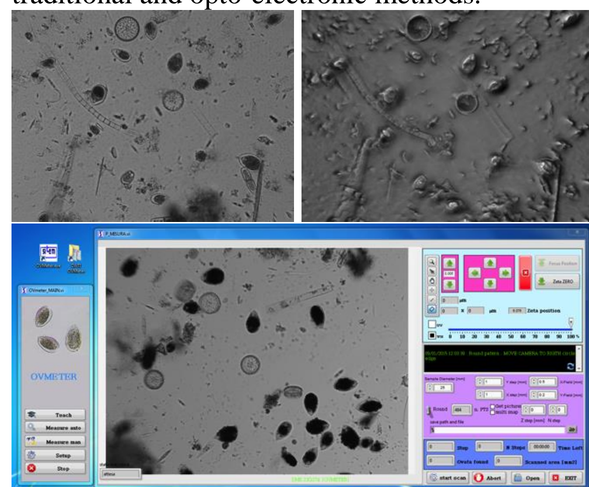

Fig.2. Enhanced 2D and 3D images obtained through QPM and screenshot of the automatic counting software.

Predictive tool for *Ostreopsis* blooms (WP5)
Ostreopsis bloom dynamics at different spatial and temporal scales and the role of different

environmental drivers seem to be complex and contradictory, with differences at basin scales (Mangialajo *et al.* 2011). A general role of increased seawater temperature in driving the blooms has arisen from descriptive (Pistocchi *et al.* 2011) and preliminary model studies (Asnaghi *et al.* 2012). In the framework of M3-HABs project, the identification of correlations between the algal blooms and the main physical-meteorological parameters on historical datasets is in progress. All project partners made available their datasets from monitoring campaigns performed in the previous years and data from different Italian regions (Liguria, Campania, Marche), France (Nice area), Monaco, Spain (Barcelona area), Tunisia (Tunis area and Gulf of Gabes) and Lebanon have been collected. Preliminary analyses on the pilot area (Ligurian Sea, Italy) have been performed in order to highlight the variables that drive the bloom dynamics. Multiple linear regression models together with more complex models, such as pure quadratic regression and neural networks, with cross validation, have been applied by CoNISMa and OnAir. A hindcast database for meteomarine and physical parameters in the pilot area, through the use of a 3D numerical model (Ligurian Sea, Italy), has been implemented by DHI Italy and will lead to the development of a predictive tool for *Ostreopsis* blooms. The tool will be able to predict expected cell abundances along the coasts (regression mode) and to give an alert when/where a given alarm threshold is probably exceeded (classification mode), in order to trigger the emergency procedures. The expertise acquired implementing the predictive tool for the pilot area will be passed to partners and students involved in the project, through the Summer School dedicated to "*Ostreopsis* bloom modelling" in Tunisia.

Common and inter-calibrated sampling strategies and protocols (WP6)

Ostreopsis occurs along Mediterranean coasts since the '90s, but bloom events causing human intoxication (via aerosol) have been recorded starting from 2004 in Spain (Vila *et al.* 2008), 2005 in Italy (Genoa; Ciminiello *et al.* 2006), 2006 in France (Kermarec *et al.* 2008) and 2009 in Algeria (Illoul *et al.* 2012).

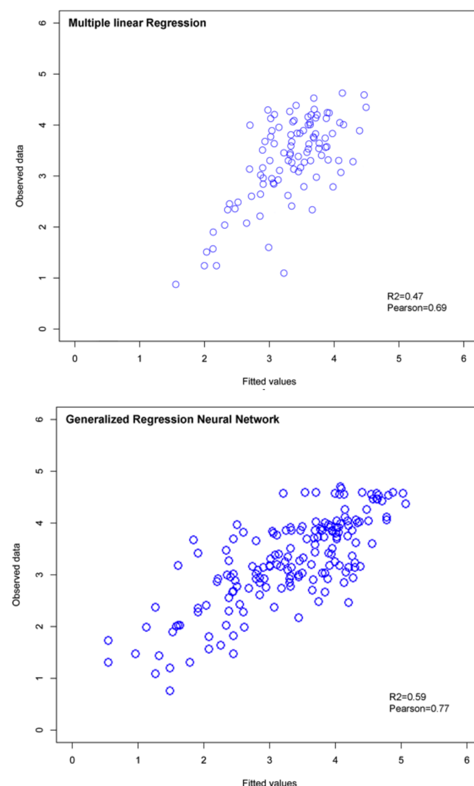

Fig. 3. Plots of *Ostreopsis* cells/l (Log10 scale) observed *versus* predicted through a multiple linear regression model and a neural network model, with related r squared and Pearson correlation coefficient, for the pilot area.

Following these events, toxic microalgal monitoring programs have been implemented systematically in many countries to assess the potential for bloom formation, guarantee the protection of human health, and ensure water quality, aquaculture and other economic activities related to coastal waters (Andersen *et al*. 2003; McGillicuddy *et al*. 2005; Miraglia *et al*. 2009; Hallegraeff, 2010). Monitoring protocols applied by the research institutes and environmental agencies across Mediterranean counties differ, making results hardly comparable. Additionally, some countries on the southern Mediterranean coasts only recently recorded, occasionally, the presence of *Ostreopsis* in planktonic samples, and they still do not have developed a specific protocol for monitoring benthic dinoflagellates. M3-HABs project will provide a common pan-Mediterranean protocol, providing an efficient tool for all interested countries and making the process mostly cost and time effective. Project partners exchanged their experience on sample collection, processing, preserving technique and counting methods during an Intercalibration Sessions that took place during the kick-off

meeting: a preliminary common and agreed sampling protocol was produced by LOV and UNS and all project and associate partners were trained during the first Summer School in Lebanon. During the first annual meeting in Nice, December 2014, sampling, processing and counting protocols have been refined following partners contributions and the need for differentiated protocols for environmental agencies and research institutes emerged. In this context, the tools that will be provided by M3-HABs project – the automatic counting system and the predictive tool – will be particularly helpful in reducing and focusing sampling and counting efforts.

Common risk detection and management strategies (WP7)

Ostreopsis blooms represent a risk not only for the environment, but also for people using the coast both for recreational and economic purposes. The public should be made aware about the risks associated with potentially toxic microalgal blooms, with a proper communication strategy, providing correct information while avoiding scaremongering. One of the aims of M3-HABs project is to collaborate closely with a wide range of local stakeholders, in order to disseminate project outputs and to inform the public about the real risks and about useful prevention measures.

A specific strategy has been implemented in order to involve the different categories of stakeholders, and to define the proper messages, instruments, targets for everyone. The sharing process will be implemented through the use of the "Open Group" of relevant stakeholders. The potential stakeholders could be regional and/or national environmental protection agencies, coastal municipalities, marine protected areas, diving centers, fishermen associations, offshore fish farms, medical institutions, touristic and bathing establishments, local newspapers and so on.

A complete manual containing practical information about algal blooms, guidelines for planning and organizing a monitoring program, instructions for correct risk management, communication strategies and concrete advice will be drafted, taking into account common points and differences between involved countries. The "Open Group" will validate these guidelines and the other in progress outputs of the project, and will share the information, or the results of the project in the different networks in which the Open Group's members are involved in.

The guidelines will be outlined with particular attention to the needs of the relevant stakeholders and will be made available to the wide public through the project website, in order to induce larger awareness of the risks associated to *Ostreopsis* blooms and improve management capacity of local authorities.

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New *Ostreopsis* species record along Cyprus coast: toxic effect and preliminary characterization of chemical-molecular aspects

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Abstract

The genus *Ostreopsis* Schmidt includes harmful benthic species that have been reported worldwide in both tropical and temperate regions. To date, genetic studies confirmed the presence of two genotypes corresponding to the morphotypes of *O.* cf. *ovata* and *O.* cf. *siamensis* in the Mediterranean Sea; recently a new genotype of *Ostreopsis* sp. was found along Greece and Cyprus coasts.
Reliable data on harmful algal blooms and related intoxication cases in the eastern Mediterranean basin are still scarce. The present study describes, for the first time, toxic effects and chemical-molecular aspects of the Cypriot genotype of *Ostreopsis* sp.
Ecotoxicological bioassays were performed exposing *A. salina* nauplii to the following treatments of cultured *Ostreopsis* sp.: untreated culture, filtered and resuspended cells in fresh medium, resuspended and sonicated cells in fresh medium, growth medium devoid of algal cells by 6 µm (mucilage remains in the treatment) and 0.22 µm mesh size filtration. Our results show higher toxic effects (% of mortality) with the whole *Ostreopsis* sp. culture ($LC_{50\text{-}48h}$ = 45 cells/ml). Given these findings, the Cypriot strain seems to be less toxic than the most widespread species *O.* cf. *ovata* ($LC_{50\text{-}48h}$ < 4 cells/ml), though further studies are needed to better understand the toxicity of this new genotype.
Preliminary LC high resolution (HR) MS studies for the Cypriot Ostreopsis sp. strain reveal a peculiar toxic profile: no palytoxin and ovatoxins so far known were not detected, while the presence of new palytoxin-like compounds was highlighted, confirming the possibility of being considered a distinct species from Mediterranean *O.* cf. *ovata*.

Keywords: Ostreopsis, toxicology, HABs, benthic dinoflagellates, Cyprus, Mediterranean Sea.

Introduction

The genus *Ostreopsis* Schmidt includes harmful benthic species that have been reported worldwide, both in temperate and tropical coastal waters. Among this genus, some species are known to produce palytoxin (PLTX)-analogs complex and large amount of mucilage that can cover the sea bottom (Shears and Ross 2010). *Ostreopsis* spp. blooms have been associated with benthic marine organism mortalities and human health concerns and these events are increasing in the Mediterranean Sea.
Recently, as more isolates from Atlantic, Mediterranean basin and Pacific areas, have been sequenced, the identification and phylogeographical characterization of cryptic species belonging to this genus is starting to become clearer. To date, genetic studies confirmed that the Mediterranean Sea hosts two genotypes corresponding to the morphotypes of *O.* cf. *ovata* and *O.* cf. *siamensis.* A new genotype of *Ostreopsis* sp. has been recently found along Greece and Cyprus coasts (Parsons et al. 2012; Penna et al. 2014), but reliable data on harmful algal blooms and associated poisoning events in the eastern Mediterranean basin are still scarce.
This study describes, for the first time, toxic effects and chemical-molecular aspects of the Cypriot genotype of *Ostreopsis* sp. obtained by a sample collected during summer 2013 in Vasiliko Bay (south Cyprus), an area characterized by a

A. Lincoln MacKenzie [Ed]. *Marine and Freshwater Harmful Algae 2014*. Proceedings of the 16th International Conference on Harmful. Algae. Cawthron Institute, Nelson, New Zealand and the International Society for the Study of Harmful Algae (ISSHA)

bad ecological status, according to a macroalgal WFD index (Orfanidis *et al.*, 2001), assessed during the previous summer and spring .

Material and Methods

Ostreopsis spp. strains were isolated from seawater samples collected from Vasiliko bay, a heavily impacted coastline at south Cyprus in July 2013; sea water temperature on the sampling day was 25 °C and salinity equal to 39 PSU. Clonal cultures were established and maintained in F/4-Si medium (Guillard *et al.*, 1975) at temperature of 23 ± 1 °C, with a standard 14:10 h light – dark cycle (photon flux of 100 $\mu E\ m^{-2}\ s^{-1}$). Subsamples of cultures were collected at the exponential growth phase by centrifugation at 4000g for 15 min. The supernatant was removed and the pellets were immediately processed or stored at -80 °C until DNA extration. DNA extration, amplification and sequencing of ribosomal genes and phylogenetic analyses were carried out as described by Penna *et al.*, (2010). *Ostreopsis* sp. C1036 strain pellet was added of methanol:water 1:1 and sonicated in pulse mode under cooling in a ice bath. The mixture was centrifuged for 5 min at 4000g and the supernatant was analyzed by liquid chromatography-high resolution mass spectrometry (LC-HRMS) and MS^2 in Full MS^2 positive ion mode according to Ciminiello et al. (2012) to characterize its toxic profile. The ecotoxicological bioassay was performed using the same algal strain which has been cultured into sterilized marine water and F/2 medium (at a concentration of 1 ml l^{-1}) and maintained at 20 °C. *Artemia salina* nauplii were exposed to 4, 40, 400 cells/ml of the following treatments of C1036 culture collected during the stationary growth phase: a) whole culture, b) filtered and resuspended cells in fresh medium, c) resuspended and sonicated cells in fresh medium, d) growth medium free of algal cells (6 µm filter mesh) and e) growth medium containing mucilage but free of algal cells (0.22 µm filter mesh). Three replicates were prepared for each combination of treatments and cell concentrations, including a control (CTR; 0.22 µm Filtered Natural Sea Water); after 48 h, the number of dead nauplii was observed under a stereomicroscope. Two way ANOVA (Factors: Concentration, 3 levels; Treatments, 5 levels) and Student-Newman-Keuls tests were performed using R statistical software. The results of the ecotoxicological bioassay are compared with those obtained in a previous study (Giussani et al. 2015) of *O.* cf. *ovata*.

Results and Discussion

The ecotoxicological bioassay recorded stronger effects with the whole and the resuspended *Ostreopsis* sp. 2 culture treatments, having LC_{50-48h} values of 45 cells/ml and 99 cells/ml respectivelly, while the other treatments showed LC_{50-48} of 365 cells/ml (in GM 6µm) and above 400 cells/ml (with GM 0.22 µm and sonicated culture) (Table 1). These data describe a toxicity pattern similar to that previously observed in the most widespred species *Ostreopsis* cf. *ovata.* In fact both species caused higher lethal effects on model organisms when a direct contact with living cells occured (Faimali et al. 2012, Giussani et al. 2015). The Cypriot genotype also presented significantly high mortality values with treatments which did not contain algal cells (54 % in GM 6 µm and 41 % in GM 0.22 µm), but only at the highest concentration tested, suggesting a different mechanism for the toxins release compared to the one that occurs in *O.* cf. *ovata*.

Table 1. LC_{50-48h} values obtained exposing nauplii of *A. salina* to several treatment of *Ostreopsis* sp.2 and *O.* cf. *ovata* cultures.

Treatments	*Ostreopsis sp.* 2 (Cyprus, C1036) **LC_{50-48h}**	*O.* cf. *ovata* (Genoa, CBA 292012) **LC_{50-48h}**
Culture	45 (31 – 63)	<4
Resuspended	99 (40 - 244)	15 (12 - 20)
Sonicated	>400	66 (51 - 84)
GM 6 µm	365 (n.c.)	-
GM 0.22 µm	>400	>400

These findings are supported by the LC-HRMS analysis, which from a qualitative stand point, reports a totally new toxic profile for the C1036 strain characterized by the presence of new palytoxin analogues, named ostreotoxin. As for the quantitative aspect, compared to *O.* cf. *ovata*, *Ostreopsis* sp.2 exihibits a lower toxin content (0.17 pg/cell) in agreement with the higher LC_{50} value.

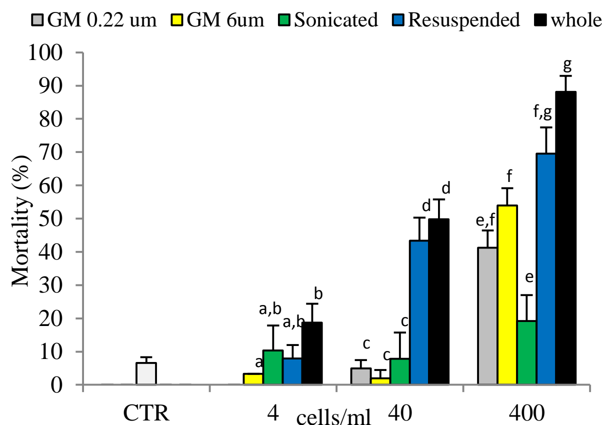

Fig. 1. Mortality (avg ± SE, N = 3) of *Artemia salina* after 48 h of exposure to 0.22 µm filtered growth medium, 6 µm filtered growth medium, sonicated *Ostreopsis* sp. 2 culture, resuspended *O*streopsis sp. 2 culture and whole *Ostreopsis* sp. 2 culture during late stationary phase of the growth curve. CTR (white bar): control in filtered natural seawater. Different letters (a-g) represent significant differences (SNK test results).

Table 2. Mono-isotopic ion peaks (*m/z*). Percentage (%) and amount (pg/cell) of new Ostreotoxins produced by the C1036 Cypriot *Ostreopsis* sp. 2 strain.

Name	$[M+H+Ca]^{3+}$ (*m/z*)	pg/cell	%
Ostreotoxin-a	909.8246	0.100	58
Ostreotoxin-b	915.1553	0.070	40
Ostreotoxin-c	920.4868	0.004	2

Phylogenetic results shows that the Cypriot strain is included in the *Ostreopsis* sp. 2 clade (David et al. 2013).

Ostreopsis sp. 2 can be considered a separate species from *O.* cf. *ovata*. Nowadays, in the Mediterranean Sea three different genotypes within the genus *Ostreopsis,* as *O.* cf. *ovata, O.* cf. *siamensis* and *Ostreopsis* sp. *2.* are recognised. Further interdisciplinary studies are ongoing to assess and characterize the genus *Ostreopsis* in the eastern Mediterranean area.

Acknowledgements

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Inorganic nutrients uptake and organic phosphorus utilization by *Ostreopsis* cf. *ovata*

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Abstract

Although nutrient implication in bloom dynamics has not yet been clarified, in *Ostreopsis* cf. *ovata* cultures the importance of nutrient concentrations (N and P) for cell growth and toxicity has been remarked. Nutrient uptake pattern in cultured strains also evidenced the need for balanced N/P ratios for optimal growth of this species; moreover it appears that the intracellular nutrient ratios tend towards balanced conditions independently by concentrations (N/P ratio) in the external medium. The present study was therefore performed in order to get better insights into nutrient utilization by *O.* cf. *ovata*. The characterization of the nutrient uptake kinetics showed that this species has higher V_{max} and higher affinity values for P uptake than for N. In addition, a study performed with cultures grown at different N/P ratios, showed that alkaline phosphatase activity was induced when external P was depleted or present in low amounts. These findings attest to the high efficiency of *O.* cf. *ovata* in both inorganic and organic P acquisition, an aspect that could confer advantages towards competing species.

Keywords: alkaline phosphatase, N/P ratio, nutrient uptake, *Ostreopsis* cf. *ovata*

Introduction

Ostreopsis cf. *ovata* blooms along Italian coasts display different characteristics for intensity, toxic effects and blooming period; it is therefore difficult to relate them to specific environmental conditions. Among the different parameters involved, nutrient influence on blooms is one of the most difficult to ascertain due either to the complexity in obtaining a large dataset of measurements and to the difficult interpretation of the data. Culture studies can thus be of help in understanding the nutrient dynamics of a microalga species. Previous studies performed in order to correlate nutrient availability, growth and toxicity of this species have left some questions open. For example: 1) which is the main limiting nutrient? P uptake occurred slightly faster than N uptake, however when N was limiting P uptake significantly decreased while when P was limiting N uptake rate remained mostly unaffected (Vanucci *et al.* 2012; 2) is toxin production influenced by cell nutrient status? Nutrient depletion did not cause any increase in toxin content per cell both under unbalanced (Vanucci *et al.* 2012 and balanced conditions (Pezzolesi *et al.* in prep.), however, cell toxin production sharply increased as soon as external nutrients were depleted (Pezzolesi et al., 2014); 3) can this species obtain nutrients from organic matter? The cells were able to maintain a constant intracellular N/P ratio despite the fast P uptake resulted in an unbalanced N/P ratio (high) in the external medium (Pezzolesi et al., in prep.; Pinna et al., submitted).

The present study was aimed to integrate previous results with new data, obtained using *O.* cf. *ovata* cultures, in order to better understand:

1) the nutrient utilization pattern,
2) the growth and cellular responses to different N/P ratios,
3) the relationship between nutrient utilization pattern and toxicity.

Material and Methods

Ostreopsis cf. *ovata* cultures were grown in 50 mL medium having nutrients of f/2 medium (Guillard, 1975) with the exception of N and P which were added in different concentrations in order to obtain five N/P ratios (16, 24, 30, 50, 100); these N/P values were achieved by adding the two macronutrients both at a low (N=11,8 µM; P= 0,74-0,12 µM) and a high level (N=176,6 µM

P= 11,0-1,76 μM). Four replicates for each condition were performed. Low nutrient cultures were grown for 8 days and those at high nutrients for 11 days. On the last day the following parameters were measured: cell numbers (Utermöhl, 1958) (n=4), alkaline phosphatase activity (as described in Cucchiari *et al.*2008) (n=2), dissolved N and P (kit analysis with DR/2010; Hach, Colorado, USA) (n=2), cellular N, C (through ThermoFisher organic elemental analyzer Flash 2000) and P (according to Menzel and Corwin 1965) (n=2).
Nutrient uptake rates were measured as described in Kwon *et al.* (2013).

Results and Discussion

Inorganic nutrients uptake
N and P uptake kinetics were studied allowing the calculation of the semi-saturation constant (K_s) and the maximal transport rate (V_{max}) for *O.* cf. *ovata*.
P uptake was characterized by higher V_{max} (mean value: 1.36 pmol cell^{-1} h^{-1}) and lower K_s (mean value: 4.82 μM) values than those measured for N (mean values: 1.02 pmol cell^{-1} h^{-1} and 8.4 μM, respectively) attesting that this species has a higher transport affinity for P than for N and that the former nutrient is taken up by cells at higher rates. This finding was in agreement with the pattern previously observed measuring the external nutrient consumption during growth (Vanucci *et al.* 2010; Pezzolesi *et al.* 2014) and gives consistency to those results. A comparison between the kinetic parameters of *O.* cf. *ovata* with those of different species evidenced that Ks values for N and P in *O.* cf. *ovata* were in the range of those observed in benthic diatoms (Kwon *et al.* 2013) but higher than those measured in planktonic toxic dinoflagellates (e.g. *Alexandrium tamarense* and *A. catenella*) (Yamamoto and Tarutani, 1999; Jauzein *et al.* 2010); conversely, the V_{max} values were higher for *O.* cf. *ovata* then for the other species and, in particular, P value was twice the average value.

Effect of N/P ratio on growth
By performing cultures under different initial N/P ratios it was observed that *O.* cf. *ovata* growth, evaluated as cell numbers (data not shown) or as carbon (Fig. 1), was affected mainly by the nutrient level rather than by the N/P ratio. Carbon concentrations were 4 to 5-fold higher in HN than in LN cultures but, within the nutrient level, values were similar among the different N/P conditions. Cultures in low nutrients (LN) reached a similar final cell concentration, not higher than 1,500 cell/mL, and stopped growing before depleting the external N and P (data not shown). Those in high nutrients (HN) reached the highest cell concentrations at N/P ratios of 24 and 30 (12,369 and 12,525 cell/mL, respectively) and only cultures with N/P ratios equal or higher than 30 depleted all the P, while N was not completely exhausted (data not shown).

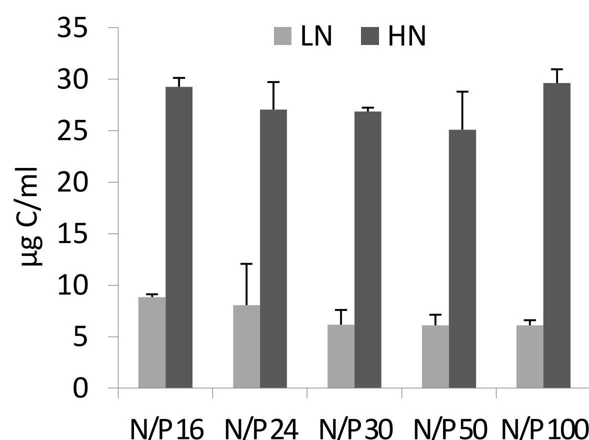

Fig. 1. Carbon concentrations measured in *O.* cf. *ovata* cultures grown in the presence of high (HN) or low (LN) nutrients levels: N and P were added in order to obtain different initial N/P ratios. The measurements were performed at day 8 (LN) or 11 (HN) of growth.

C, N and P contents in *O.* cf. *ovata* cells were measured in the last day of growth (day 8 and 11 for LN and HN, respectively), which represents the beginning of the stationary phase, and allowed the corresponding C/N and C/P values to be obtained. Cell C/N ratios (Fig. 2a) remained stable and displayed higher values in cultures grown with lower than those with higher nutrients content, presumably due to an increased polysaccharide production. Cell C/P (Fig. 2b) and N/P (Fig. 2c) ratios increased only in HN cultures which grew at higher levels and which depleted the added phosphate faster; both ratios increased in cultures having an external N/P ≥30 and increased with the increasing of P-depletion. These results attest that the best condition for *O.* cf. *ovata* growth is represented by the presence of high macronutrient levels in a balanced ratio, between 16 and 30. Nitrogen seems to be the most important element for high biomass accumulation while the P stored, thanks to high uptake capacity, can sustain growth when the environment becomes P-limited.

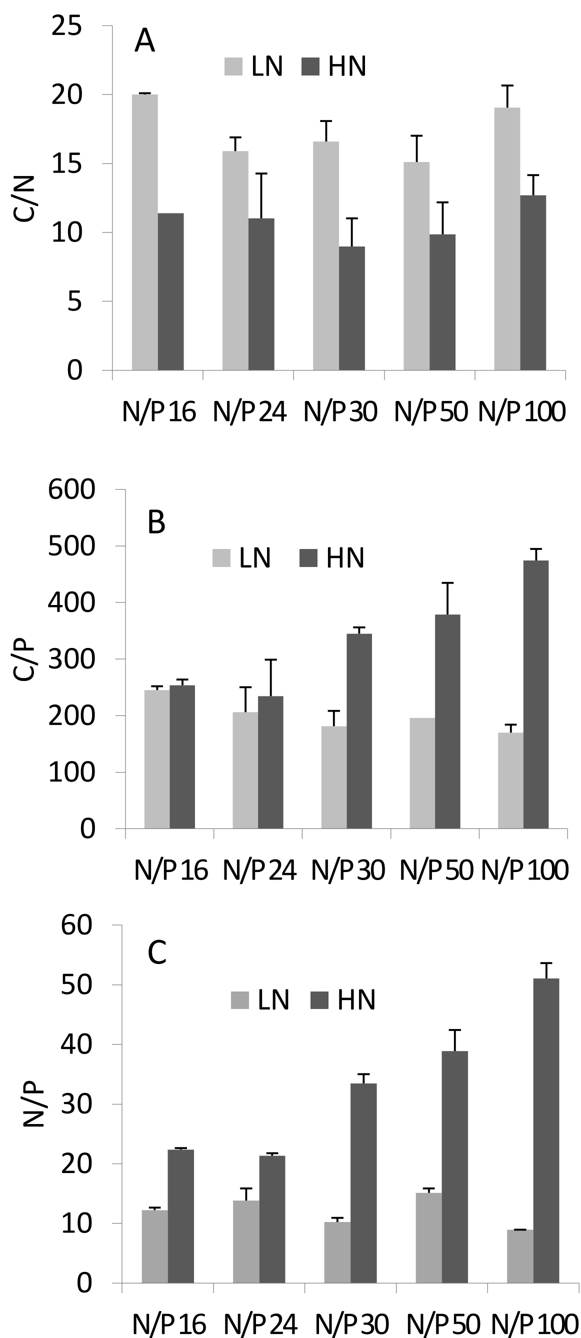

Fig. 2. Cell C/N (A), C/P (B) and N/P (C) ratios measured in *O.* cf. *ovata* cultures grown in the presence of high (HN) or low (LN) nutrients levels, at day 8 and 11, respectively.

Organic phosphate utilization

Alkaline phosphatase activity (APA) showed the lowest (and comparable) values in cultures with N/P = 16 and 24, both at low and high nutrient levels (Fig. 3). The enzyme activity was induced in cultures with a N/P ratio higher than 24 both in HN and LN conditions. The enzyme was induced also in LN cultures which did not deplete all the external P and where the intracellular C/P and N/P ratios were not as much affected as in HN cultures (Fig. 2b,c). This is consistent with the observation (Jauzein *et al.* 2013) that intracellular P concentrations are not as direct in APA regulation

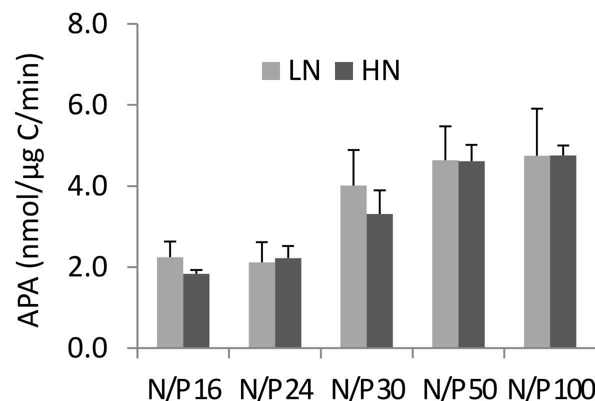

Fig. 3. Alkaline phosphatase activity measured in *O.* cf. *ovata* cultures grown in the presence of high (HN) or low (LN) nutrients levels; N and P were added in order to obtain different initial N/P ratios. The measurements were performed at day 8 (LN) or 11 (HN) of growth.

as are the extracellular concentrations. In addition, in LN cells a pgP pgC^{-1} value, very close to the threshold value of 0.016 reported as necessary for AP synthesis (Jauzein et al. 2013), was observed. In HN cultures the enzyme was induced in the culture condition where the external P was firstly depleted. In a preliminary experiments, where APA was measured at day 8 also in HN cultures, any APA induction was observed (Pistocchi, unpublished). Thus the possibility to use organic P as soon as the inorganic form is depleted attests the high capacity of *O.* cf. *ovata* to scavenge this nutrient from the environment, an aspect that, in addition to highly efficient uptake parameters, could confer a competitive advantage towards other microalgae species.

In the attempt to establish a link between the present results and *O.* cf. *ovata* toxin production, it has been recently observed (Pinna *et al.*, submitted) that an increase in cellular C/P and C/N ratios along the growth curve, due to an ongoing primary production coupled with poor nutrient conditions, corresponds to an increase in toxin production (increasing tox:C ratio), as it has usually been observed in the stationary phase. The present results allowed us to ascertain that the presence of high initial nutrient levels, characterized by N/P ratios higher than the optimal ones (e.g. for nitrogen inflow), can sustain

high cells growth determining a high C/P ratio in short time. On the other hand, in the presence of low nutrient levels, C/N values higher than those considered as physiological were observed. Both conditions could thus be linked to an increased relative cell toxin production.

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Growth promotion of *Heterosigma akashiwo* by marine microorganisms; implication of marine bacterium in bloom formation

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Abstract

The ecophysiological importance of bacterial assemblages that associate with algal blooms, which is termed 'phycosphere', is beginning to be acknowledged. However, information regarding implication of bacterium in HABs is still limited. Here, we attempted to screen marine bacterium that associate with and affect the physiology of *Heterosigma akashiwo* (*Ha*), a HAB forming raphidophyte. To this end, six axenic *Ha* strains were established and several marine bacteria isolated to form laboratory maintained non-axenic cultures. One bacterial strain, *Altererythrobactar ishigakinesis*, facilitated growth and increased culture yield of *Ha*. In addition, the bacterium increased significantly along with *Ha* growth, suggesting that the two organisms are in symbiotic relationship. Two other bacterial strains, *Winogradskylla poriferorum* and *Spongiibacterium flavum*, increased the *Ha* culture yield in *Ha* isotype specific manner. These data indicate the importance of commensal bacteria in *Ha* physiology.

Keywords*: Heterosigma akashiwo*, marine bacteria, symbiosis

Introduction

Heterosigma akashiwo (*Ha*) is one of the noxious raphidophyte species which often forms HABs during summer, particularly in coastal areas. While several reports described marine bacteria that kill *Ha* (Skerratt, et al., 2002, Nagasaki, et al., 2002, Imai, et al., 2001, Kim, et al., 1998, Lovejoy, et al., 1998, Yoshinaga, et al., 1998, Kim, et al., 2007, Liu, et al., 2008, Kim, et al., 2009, Kim, et al., 2009, Park, et al., 2010, Cho, 2012), information about growth promoting marine bacteria is limited (Liu, et al., 2008). To gain more information about *Ha*-bacterium interaction, we generated axenic *Ha* strains and isolated bacteria associating laboratory maintained *Ha* cultures. In this study, three bacterial strains were found that facilitate *Ha* growth. This study suggests the potential importance of associated bacteria for *Ha* bloom status.

Material and Methods

Ha strains used for this study

Ha isolates, H93616 (isolated from Uranouchi Bay, Kochi Prefecture, Japan), NEPCC522 (isolated from Jericho Beach, BC, Canada), *Ha*Fk01 (isolated from, Fukuoka Bay, Fukuoka prefecture, Japan), *Ha*00-17 (isolated from Hiroshima Bay, Hiroshima prefecture, Japan), *Ha*GS95 (isolated from Gokashi Bay, Mie Prefecture, Japan 1995), and *Ha*Tj01 (isolated from Tajiri Bay, Hiroshima prefecture, Japan), were used for this study. Algal strains were maintained in modified SWM3 medium (Yamasaki, et al., 2007) in an environment-controlled chamber with a photoperiod (12 h of 100 µmol m^{-2} s^{-1} light/12 h dark) at 25°C.

Ha axenization

The antibiotic treatment was conducted in stepwise manner. Briefly, penicillin and streptomycin were added (100 units/mL and 100 µg/mL respectively), to the raphidophyte culture with concentrations of 2 to 7 $\times$ 10^4 cells/mL. When the culture density reached 5 $\times$ 10^5 to 10^6/mL, they were diluted to 1/20 in the media with the two antibiotics, and then maintained until 5 $\times$ 10^5 to 10^6/mL. These steps were repeated with the addition of ampicillin to 100 µg/mL, followed by the addition of kanamycin to 60 µg/mL.

To check if there were any bacteria in the raphidophyte cultures, soft-agar media was prepared by adding 0.2% of bacto-agar to commercial Difco™ Marine Broth 2216. The 100 µL of raphidophyte cultures with cells at the density of 5 $\times$ 10^5 to 10^6/mL were stab-inoculated to the soft-agar media using long pipetman tips by piercing into the media to at least a 5 cm depth. The tubes were incubated at 25 to 28°C in

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darkness for at least 3 weeks, and the growth of the microorganisms was assessed.

Isolation of *Ha* associating bacteria
When the bacteria in the soft-agar media became visible, they were streaked on Marine Broth solid media containing 2% of agar, and incubated at 25°C under aerobic and microaerobic conditions. When colonies appeared, the 16S rDNA sequence was amplified and squenced by standard techinques, and the sequence information was used for identification of bacteria. The bacteria were cultured in liquid Marine Broth for co-culture experiments.

Ha-bacterium co-culture
For co-culture experiment, the bacterial strains were precultured in Marine Broth 2216, and the 600-μL of the bacterial culture adjusted to the OD_{600}=0.01 was added to the 60-mL of *Ha* cultures adjusted to 5×10^3 cells/mL.
For *Ha* cell enumeration, a Mini Automated Cell Counter MoxiZ (E.I Spectra, LLC, Hailey, ID, USA) was used according to the manufacturer's instruction. The number of bacterial cells in the culture was estimated as colony forming units.

Results and Discussion

Ha strains were successfully rendered bacterium free by the step-wise antibiotics treatments. Importantly, removal of associating microorganisms affected growth rate and maximum culture density of some *Ha* isolates. Figure 1a-c show the growth rates of bacteria-free and non-axenic strains of *Ha,* both in growth medium without any antibiotics. The propagation of the H93616 non-axenic strain was significantly faster than the axenic strains during its logarithmic growth rate (Figure 1a). In addition, culture yield, or the maximum cell density, of the non-axenic culture was more than double of that of bacterial-free H93616 (Figure 1a). Similarly, the non-axenic NEPCC522 strain shows markedly faster propagation and higher culture yield compared to its bacteria-free counterpart (Figure 1b). These observations suggest that these non-axenic *Ha* cultures may contain growth facilitating and/or nutrition recycling bacteria. In the case of *Ha*Tj01 strains, the growth of the non-axenic strain was almost equal to or slightly slower than that of the axenic strain (Figure 1c). These observations provide additional proof for the importance of the effects of commensal bacteria on raphidophyte physiology.

Several bacterium strains were isolated from the non-axenic cultures. Among them, three strains, *Altererythrobactar ishigakinesis, Winogradskylla poriferorum* and *Spongiibacterium flavum*, had an effect on *Ha* growth. The strain identified to be *A. ishigakinesis* promoted growth of all the tested *Ha* isolates. When *A. ishigakinesis* was added to the

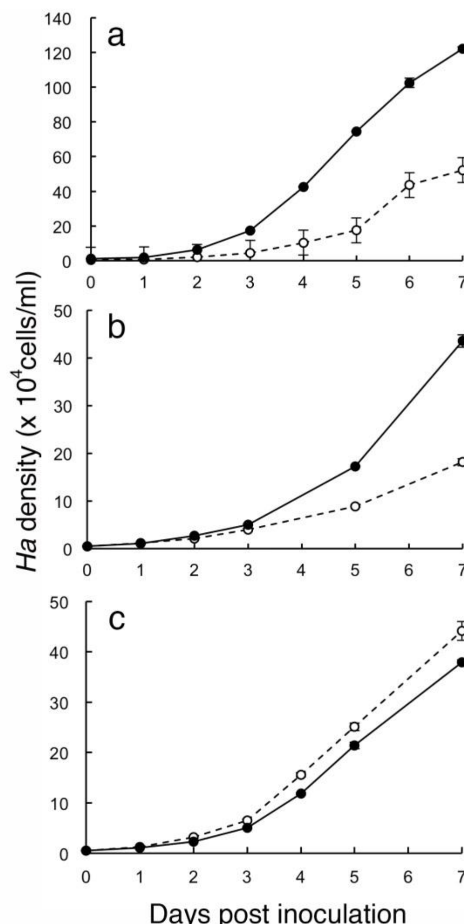

Fig.1. Comparison of the growth rate of non-axenic and axenic strains of *Ha*. a: H93616, b: NEPCC522, and c: *Ha*Tj01. Open circles with broken lines: axenic strains, closed circles with solid lines: non-axenic strains. Data points represent the average ± standard deviation (SD) of triplicates.

bacteria-free *Ha* cultures (Figure 2a-c), the *Ha* growth rate was facilitated, and the culture yield was also markedly increased in all the tested isolates. Importantly, *A. ishigakinesis* increased during the growth phase of *Ha* in the culture, whereas the population collapsed after the *Ha* culture densities reached their plateau. When *A. ishigakinesis* alone was inoculated in artificial seawater, the bacterium did not propagate as it had in the presence of *Ha.* These observations

demonstrate that *Ha* and *A. ishigakinesis* are in mutualistic relationship.

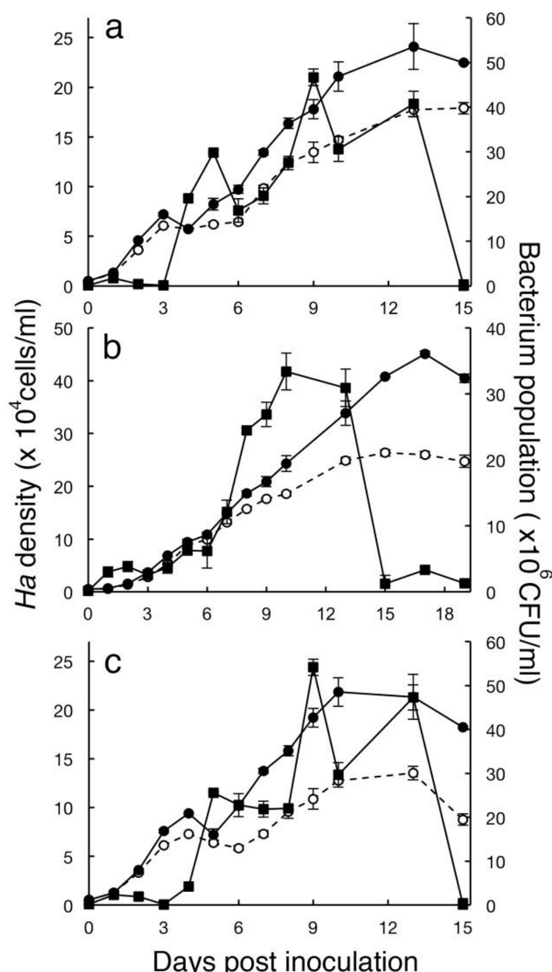

Fig. 2. Growth promotion of different strains of *Ha* by three bacterial strains, *A. ishigakinesis. Ha* strains H93616 (a), NEPCC522 (b), and *Ha*Fk01 (c) were cultured with (closed circles with solid lines) or without (open circles) *A. ishigakinesis*. Propagation of the bacteria added to the culture (indicated on left) was evaluated as colony forming unit (black squares with dashed lines). The data are mean ± SEM of triplicate measurements.

Interestingly, two other bacterial strains, *S. flavum* and *W. poriferorum,* promoted *Ha* growth in isotype specific manner (Fig. 3). When *S. flavum* was added to a culture of the *Ha*Tj1 strain, the increase in *Ha* growth rate was insignificant; the maximum culture density was similar to the control (Fig. 3c). However, the effect of *S. flavum* on the maximum culture density of two *Ha* strains, H93616 and *Ha*Fk01, was minimal (Fig. 3a & b). Similarly, when *W. poriferorum* was added to *Ha*Tj1 culture, the maximum culture density was increased compared to control (Fig 3f), while only minimal effect was observed on the growth of H93616 and *Ha*Fk01 (Fig3d&e). Regardless of the differential effect on *Ha* growth, the number of bacterial cells in the different cultures of bacterial strains propagated throughout the period of experiments was comparable (data not shown), suggesting that the difference in the propagation of bacteria did not account for their different strain effects on maximum *Ha* densities.

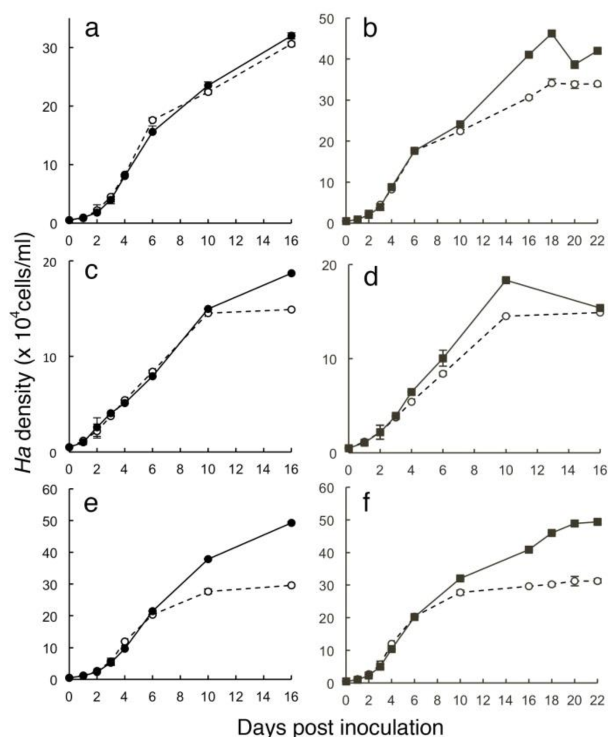

Fig. 3. Effect of *S. flavum* (a-c) and *W. poriferorum* (d-f) on growth of H93616 (a & d), *Ha*Fk01(b & e), and *Ha*Tj1 (c & f). The *Ha* cultures were maintained with (black square) or without (open circle) *S flavam* or *W. Priferorum.* The data are mean ± SEM of triplicate measurements.

From this study, we identified three *Ha* growth-promoting bacterial strains. Importantly, *A. ishigakinesis* stimulated growth of all the tested *Ha* strains, while *S. flavum* and *W. poriferorum* increased only one *Ha* isolate. These observations suggest the importance of commensal bacteria as well as *Ha* isotype for understanding *Ha*-marine bacterium interactions.

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Ohara Foundation for Agricultural Research, and Japan Society for the Promotion of Science. We thank Dr. Shikata (Fisheries Research Agency, Japan) for providing us with the *Ha* isolate.

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Modelling the potential impact of *Alexandrium catenella* in a marine farming area

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Abstract

The emerging issue of saxitoxin contamination of shellfish associated with *Alexandrium catenella* in a major aquaculture region of New Zealand has led us to investigate tools that will help forecast and communicate the risk of major events. Over the last five years a variety of conditions have been observed. In some years the effects of the annual *A. catenella* bloom have been minor, in other years the wide-spread occurrence of high cell concentrations have led to the closure of important shellfish growing areas. An overview of biophysical observations is presented, accompanied by two preliminary model simulations undertaken for a small embayment commonly associated with large aggregations of *A. catenella*.

Keywords: Alexandrium catenella, aquaculture, modelling, paralytic shellfish poisoning

Introduction

Paralytic shellfish toxin (PST) contamination due to blooms of *Alexandrium minutum* and *Alexandrium catenella* are relatively common on the east coast of the North Island, New Zealand (MacKenzie, 2014). However, they have only become a problem in the major shellfish cultivation regions of the South Island since a bloom of *A. catenella* was first detected in Queen Charlotte Sound (QCS) in late summer 2011 (Harwood *et al.* 2013). This event caused a three month suspension of commercial shellfish harvesting in the area and resulted in financial losses to shellfish growers. While cells were widespread around the sound, synoptic sampling established that the highest concentration of cells in the water column, and resting cysts in the sediments, were located in a bay (Opua Bay) at the head of an enclosed inlet extending off Tory Channel (Fig. 1). Tory Channel is a deep, well mixed, high nutrient body of water through which high velocity tidal flows exchange large volumes of water between Cook Strait and inner QCS.

Initially it appeared that the 2011 bloom was the result of a new incursion of this dinoflagellate into the Marlborough Sounds. However subsequent research has shown that high numbers of *A. catenella* resting cysts (1-2 x10^7 cysts. m^{-2}) persist in the surficial sediments of Opua Bay, and cyst counts and isotope dating of sediment core strata have shown this seed bed has been established for many decades. Future PST contamination episodes in the region are inevitable. To assist shellfish growers, we have been exploring the use of numerical models as tools to predict and plan for the likely impact of *A. catenella* blooms on their operations.

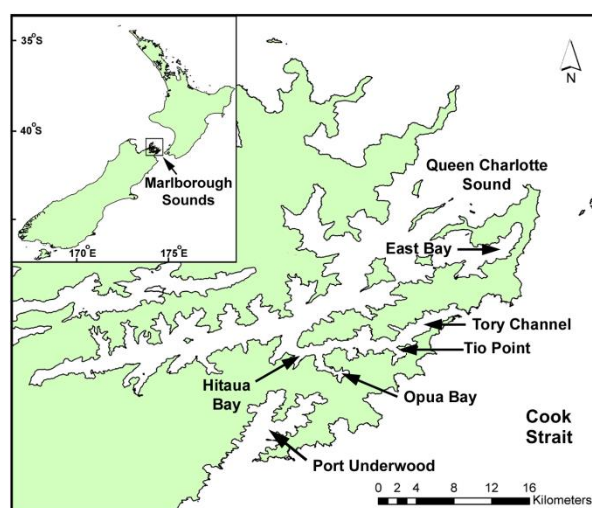

Fig. 1. Map showing locations in Queen Charlotte Sound

Two separate models have been used in these investigations: a biological model relevant to bloom dynamics in Opua Bay, and a dispersal model of the wider QCS region. The aim of the biological model is to investigate the chemical, biological and environmental processes that drive bloom formation and forecast when blooms developing within Opua Bay are likely to lead to PST contamination in the wider sound. The dispersion model aims to predict the spread of the blooms to enable shellfish farms throughout the sound to plan for the likely timing and duration of closure periods.

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Biophysical observations

Since 2011, routine weekly phytoplankton sampling and monitoring of seawater temperature at six depths through the water column in Opua Bay has been carried out between December and March each summer. Blooms of *A. catenella* occur every year but there are considerable year to year variations in their timing, magnitude and duration (Fig. 2). From these data a conceptual model of the *A. catenella* bloom dynamics has been developed.

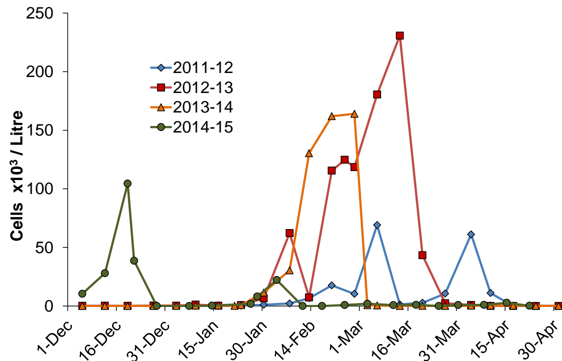

Fig. 2 Mean cell numbers of *Alexandrium catenella* in the Opua Bay water column, during bloom seasons Dec-Apr 2011-2015.

The existing cyst bed, and its replenishment each season, the hydrodynamics of the inlet, thermal stratification and the supply of inorganic nutrients from Tory Channel are the main factors that drive the annual *A. catenella* bloom. The effects of tides, winds and solar insolation on circulation and water column stratification have important impacts on the timing, intensity and duration of blooms in Opua Bay and the role that it plays as an incubator from which cells are dispersed to other areas of Queen Charlotte Sound. We have no direct measures of the conditions that initiate cyst germination except that increasing numbers of motile cells appear in the water column when bottom-water temperatures exceed 15°C. Because the water within Opua Bay originates from Tory Channel and is little affected by freshwater runoff, salinity only plays a minor role in maintaining water column stratification. Monitoring of water column thermal structure has revealed that the *A. catenella* blooms are particularly sensitive to transient water column mixing events brought about by high winds and tide range excursions. All the blooms so far documented have been rapidly terminated as a result of tidal and wind events that have led to a breakdown of water column stratification. In some seasons (e.g. 2012-13 & 2013-14) stratification has been maintained for long periods and substantial blooms have developed. In other years (e.g. 2011-12 & 2014-15) repeated mixing has largely supressed bloom development.

Biological Model

The preliminary biological model has been constructed using the cloud-based modelling package insightmaker (http://insightmaker.com) with a 4th order runge-kutta solver used to ensure accurate numerical solutions. The structure of the biological box model of Opua Bay broadly follows that of the *A. fundyense* model for the Gulf of Maine described by Stock *et al.* (1995).

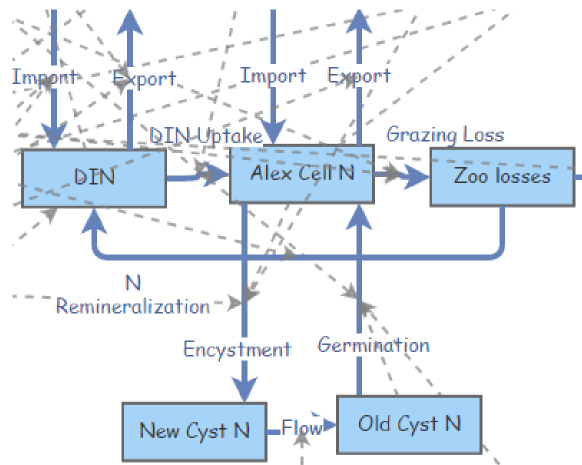

Fig. 3. Processes included in the *Alexandrium catenella* insightmaker model for Opua Bay. Blue boxes indicate stocks of nitrogen (N) mass associated with either dissolved inorganic nitrogen (DIN), *A. catenella* cells (Alex Cell N), accumulated losses to zooplankton (Zoo losses) and immature (New Cyst N) and mature (Old) cyst populations. Solid arrows indicate flows of N between stocks, dotted lines relate to parameters used in the model (not shown).

Some local customizations to the model of Stock *et al.* (1995) have been applied; for example, nitrogen limitation, flushing and possible grazer limitation have been added (Fig. 3). The model is available at this website address: https://insightmaker.com/insight/6809).

The system of equations governing the populations of available dissolved nitrogen (*N*), *A. catenella* cells (*P*), zooplankton losses (*Z*) and immature (C_{new}) and mature cyst (C_{old}) populations are described in nitrogen mass units through equations 1 & 2.

$$\frac{dN}{dt} = \frac{N_{in}}{dt} - \frac{N_{out}}{dt} - q.(\mu - g).P + g.M.P$$

$$\frac{dP}{dt} = (\mu - g).P - \frac{C_{new}}{dt} + \frac{h.C_{old}}{dt} + \frac{P_{in}}{dt} - \frac{P_{out}}{dt}$$

$$\frac{dZ}{dt} = g.P(1 - M)$$

Where possible literature sourced values have been used, but the model is at present uncalibrated. Zooplankton grazing (g) of cells is set at a constant 5% per day, with 50% of grazed N remineralised (*i.e.* M = 0.5). Cyst germination rate (h) is specified with a monthly rate, biased towards spring/summer hatching up to a maximum of 1% of the mature cyst population per day (e.g. Ishikawa *et al.* 2015). Encystment occurs at a constant rate of 0.01 day^{-1} and cells are added to an immature cyst population (New Cyst N; Fig. 3; e.g. Ishikawa *et al.* 2015). Ageing of cysts occurs at a rate of 5% of the immature cyst population per day over the spring/summer months (1st September until 1st May). Growth (μ) is modelled as the lower of light-limited (μ_{PAR}), or nutrient-limited (μ_N) growth which are described by the following equations:

$$\mu_{PAR} = (\mu_{max} + R).(Tanh(\varepsilon.PAR_z/(\mu_{max} + R))) - R$$

$$\mu_N = \mu_{max}.N/(K_{1/2} + N)$$

Where the half-saturation constant for nutrient uptake ($K_{1/2}$) is 42 mgN/m^3 and respiration rate (R) is 0.2 day^{-1}. The temperature (T) limited maximum growth rate (μ_{max}) and photosynthetically active radiation at depth (PAR_z) are calculated as:

$$\mu_{max} = 0.56 * (- 0.000513\ T^3 + 0.0160\ T^2 - 0.0867\ T + 0.382)$$

$$PAR_z = 0.5\ PAR_0\ .\ e^{-\alpha z}$$

Idealized seasonal environmental forcing (e.g. PAR, water temperature, DIN inputs etc.) and initial conditions for biological parameters and nitrogen concentrations have been used for feasibility testing of the model. In future, these are planned to be replaced by forecasted environmental data and *in situ* measurements.

Dispersal Model

The dispersal model is a physical model with neutral, passive tracer concentrations used to model the transport of *A. catenella* cells from Opua Bay. Transport of *A. catenella* cells is modelled using an eularian upwind advection scheme within the Semi-implicit Eulerian-Lagrangian finite-element (SELFE) hydrodynamic model of Zhang and Baptista (2008). Water within the model is redistributed by incorporating aspects of the real-world systems (e.g. bathymetric information, forcing by tides and wind, solar heating effects etc.). The model transports water and other constituents (e.g. salt, temperature, turbulence) through the use of triangular volumes (connected 3-D polyhedrons) of varying size and is described as an unstructured finite element model.

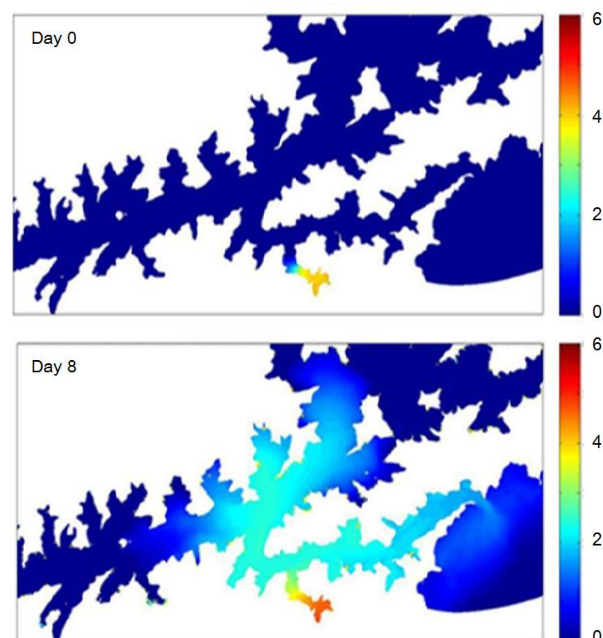

Fig. 4 Dispersion modelling results showing the simulated spread of *A. catenella* in surface waters of Queen Charlotte Sound under the influence of tidal currents from cells propagating in Opua Bay. Units displayed on the colour scale are in $\log_{10}$(cells/litre).

The unstructured modelling approach employed by SELFE allows the complex waterways of QCS to be accurately reproduced (approximately 10m resolution at the shore) and the semi-implicit approach offers large performance and stability advantages in a region with shallow waters. Validation of the model performance showed it generally performed well at producing the timing and heights of the major astronomic tides at four locations throughout the sound and current speeds at five locations (Knight & Beamsley, 2012). Although the model has not specifically been validated in Opua Bay, it has been compared to nearby sites and is deemed suitable. Initial concentrations (1x10^6 cells/L) from the climax of the March 2013 bloom (Fig. 2) were used to initialise the model. A conservative population doubling time of 5 days was used to estimate an injection rate of cells into the Opua Bay system. This growth rate was chosen as a medium approximation of observed net *in situ* population

doubling times (3-8 days) during successive blooms (MacKenzie *et al.* 2014).

Discussion

Biological modelling for Opua Bay over a two year period yields feasible results for both the *in situ* cell, dissolved N concentrations and seabed cyst populations, with vertically integrated peak bloom cell counts and timing broadly representative of observed results (Figs. 2 & 5).

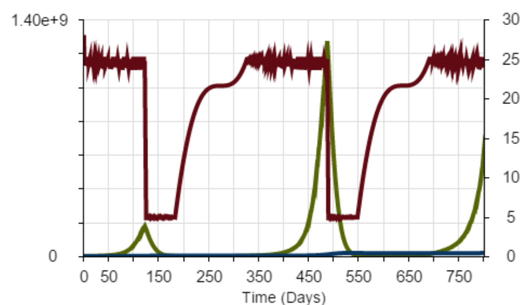

Fig. 5 An output of *A. catenella* model in Opua Bay. The left y axis refers to cell (green line) and cyst (blue line) concentrations in cells/m^2. The right hand y axis refers to DIN concentrations (red line) in mg N/m^3. The simulation begins in the southern hemisphere winter (June).

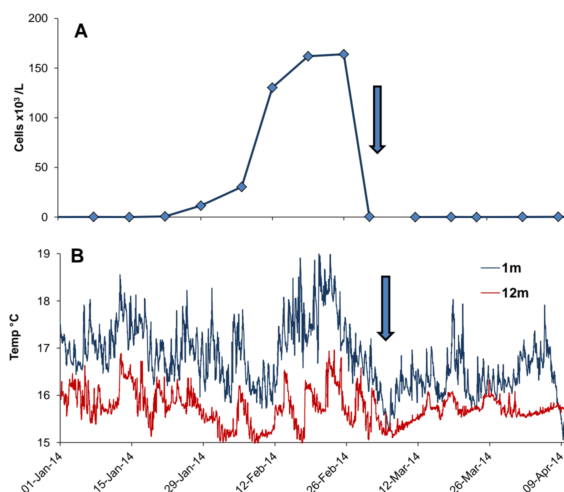

Fig. 6 *A. catenella* cell concentrations (A) and surface and bottom temperatures (B) in Opua Bay. Arrows indicate a de-stratification event associated with a rapid reduction in cell numbers.

However, reproduction of the magnitude of observed bloom events in the model is very sensitive to the retention of cells in the region. A 1% increase in the fraction of cells exported leads to a greater than ten times reduction in the peak cell concentrations. This suggests that hydrodynamics in the region play a critical role in both the movement of cells and nutrients. Empirical evidence for the importance of flushing flows in the region is present in the analysis of cessation events from *in situ* data. The events show abrupt reductions in cell concentrations associated with mixing events (Fig. 6). Consequently, while biological drivers can explain broad seasonal changes, hydrodynamic forcing appears to be an important intra-seasonal driver. Separate physical dispersion modelling of a large simulated bloom event also shows that cessation events within Opua Bay have the potential to transport cells throughout the sound (Fig. 4). Presently there is no biological simulation in the hydrodynamic model, so these estimates are likely to be lower than what would be expected in reality, as cells could continue to grow. The work presented here is still progressing, but these initial results highlight the value of building and comparing relatively simple theoretical models to data collection to improve knowledge in an area. In future it will also contribute to targeted field studies and extrapolation of findings for wider management use.

Acknowledgements

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Mating compatibility and encystment characteristics of *Alexandrium catenella* dinoflagellate strains from Chilean southern fjords

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Abstract

Resting cysts play an important role in the origin and initiation of *Alexandrium* dinoflagellate blooms in different coastal environments. *Alexandrium catenella* causes major economic impacts for the Chilean shellfish and fish farm industries, but the environmental conditions that regulate the encystment timing and cyst production rate in the seasonally variable fjords are poorly understood. We investigated mating compatibility and genetic relationships among 10 Chilean strains of *A. catenella* Group 1 using rDNA-ITS and LSU-rDNA sequences. Strain crossing experiments in all possible pairwise combinations showed variable reproductive compatibility among strains with high compatibilities among northern (Los Lagos) and southern (Magallanes) but not central (Aysén) strains. However, these mating differences were not reflected in the identical LSU-rDNA and rDNA-ITS sequences observed among the strains. Encystment was synchronized in all pairwise crosses, with cyst production starting 26 days and terminating 45 days after inoculation. Cyst production was variable among crosses ranging from 7±1.3 to 316.9±20.5 cysts ml^{-1}. The successful pairwise crosses performed with strains from the northern Chilean fjords do not explain the low concentrations of cysts detected in this area. Instead, these low cyst concentrations might be the result of local circulation patterns leading to a net flux offshore.

Keywords: Alexandrium catenella, mating compatibility, genetic analysis, encystment, Chilean fjords.

Introduction

Alexandrium catenella is a much studied dinoflagellate species in the Chilean southern fjords because of its production of saxitoxin and analogues responsible for Paralytic Shellfish Poisoning (PSP). Despite the deleterious effects on the local aquaculture, little is known about its ecology in this complex ecosystem. Several ecological studies have pointed out the importance of resting cysts in providing an inoculum for *Alexandrium* outbreaks (Anderson et al. 1987; Hallegraeff et al. 1998, Kremp, 2001; Genovesi et al. 2009). At the end of a bloom, and generally under nutrient limitation, vegetative growth ceases and cells undergo sexual reproduction (Pfiester & Anderson, 1987), and the swimming zygote resulting from the sexual fusion of gametes, ultimately become a dormant resting cyst.
In many dinoflagellate species, resting cysts are produced when compatible clones are mated (Destombe & Cembella, 1990; Blackburn et al. 2001; Figueroa et al. 2007). Thus, beside the environmental factors, mating system complexity (Kremp, 2013) and the genetic diversity in a bloom (Dia et al. 2014), might control the shifting patterns between benthic and planktonic stages. Aguilera-Belmonte et al. (2011) and Varela et al. (2012) reported high genetic diversity among Chilean strains from the southern fjords, which could potentially play an important role in gamete recognition and further cyst formation.
Other studies carried out in Chile have shown that the maximum amount of cysts in sediment does not exceed 221 cysts cm^{-3} (Seguel & Sfeir, 2003), which is much lower that the concentrations found by Yamagushi et al. (2002) in Tokuyana bay, Japan (>8000 cysts cm^{-3}). These low cyst concentrations in the northern areas of the fjords (41-46°S), do not match the massive outbreaks of *A. catenella* recorded in the last years during austral summer (i.e. February-march 2009). An important question thus arises: is the scarcity of cysts in the fjord area due to a low production of hypnozygotes after a bloom perhaps the result of low compatibility among the gametes?
The aim of this study was to determine mating compatibility and cyst production success among 10 *A. catenella* Chilean strains and explore their potential genetic relationships.

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Material and Methods

Origin and maintenance of cell cultures

Monoclonal cultures of the toxic dinoflagellate *A. catenella* isolated from the Chilean southern fjords (Fig. 1) were grown in L1 medium (Guillard and Ryther, 1962) at 17°C in sterile filtered (0.22 µm) seawater (30 psu) at 100 µmol photon m^{-2} s^{-1} (cool white fluorescent lamps) under a 12:12h light:dark cycle.

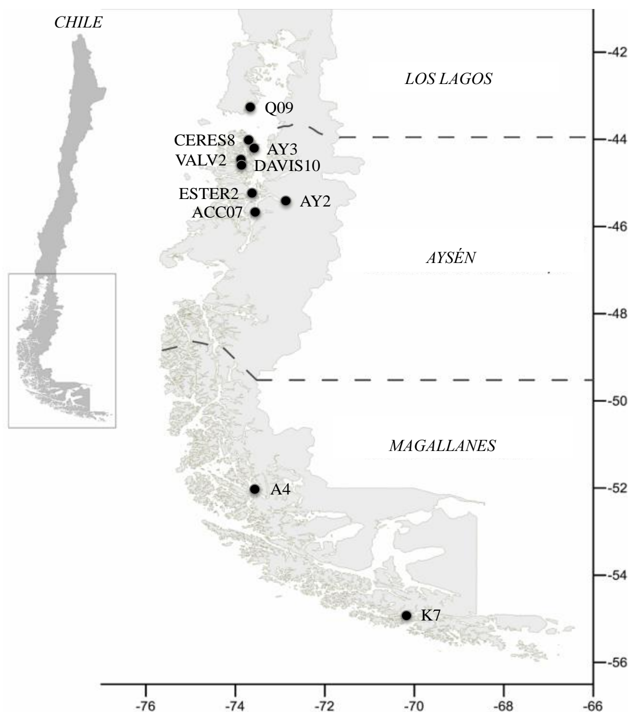

Fig. 1. Study area showing the geographical origin of 10 *A. catenella* strains isolated from the Chilean fjords.

Mating compatibility and encystment

For mating compatibility observations, 10 strains were crossed in all possible combinations in duplicate (even self-crossed controls for testing homothallism) by pipetting a cell concentration adjusted to 400 cell ml^{-1} as inoculum of a late-logarithmic-phase culture of each strain into 5 ml of L1 medium without nitrate and phosphate ($L1^{-N\text{-}P}$) in presterilized polystyrene 6 wells-plates. Plates were sealed with ParafilmTM and incubated at 17°C, salinity 28%, 100 µM m^{-2} s^{-1} (12L:12D) white fluorescence light. Plates were checked once a week for resting cyst formation by scanning the entire well (by transects) with a Zeiss Axiovert 25 inverted microscope. Each cross was scored after the cyst production (45 days after inoculation). To estimate the cyst production rate (cyst d^{-1}), 9 pairwise crosses where quantified periodically.

A square symmetrically crossing matrix was used for mating group analysis (Fig. 2). To evaluate differences in mating among the strains, a measure of strain reproductive compatibility (RC) was calculated from the scores of the crossing matrix according to Blackburn et al., (2001):

$$RCs = CIs * AVs \qquad \text{Eq. 1}$$

Where:

Compatibility index (CIs): the number of successful crosses resulting in a score of ≥ 1 divided by the total number of possible crosses.

Average vigour (AVs): the average of the number of cysts produced per cross in successful crosses involving a particular strain.

Mating compatibility and encystment were subjected to an analysis of variance (ANOVA). Normality and homogeneity of variances were assessed by the Kolmogorov-Smirnov method and Levene's test. A post-hoc analysis using a Tukey HSD test was performed to determine differences among treatments.

DNA sequencing

Total DNA was extracted from 10ml of exponential phase cultures using a MoBio PowerSoil DNA extraction kit (MoBio, USA) according to the manufacturer's protocols, diluted to an approximate concentration of 10 ng µl^{-1}, and used as template for PCR. The D1-D2 region of the LSU-rDNA was amplified as using primers D1R and D2C as described by Scholin *et al.* (1994), and the rDNA-ITAS region amplified using primers ITSA and ITSB as described by Adachi et al. (1996). Resulting sequences were corrected by visual inspection and aligned with related GenBank sequences, and neighbour-joining phylogenetic trees constructed from Tamura-Nei distance matrices using the software Geneious R7 (Biomatters, NZ).

Results and Discussion

Mating and encystment processes

Based on simple binary heterothallism (+)/(-) previously claimed for Japanese *Alexandrium catenella* (Yoshimatsu 1984), we initially attempted to categorize all strains into two groups, (+) and (-). Nevertheless, evidence of five mating groups was obtained after manual sorting and assessment of successful pairwise crosses among all strains. The matrix of intercrossing and self-

crossing compatibility is presented in Fig. 2. The intercrossing experiments showed that 53.3% (24 of 45, n=3) of the total crossing attempts (no self-crossing included) resulted in cell recognition. Homothallism among the strains was not observed. Besides the A and B mating groups, which were comparable to the groups expected in a simple (+) and (-) mating system, and group C (not compatible with groups A and B), groups D and E (strains CERES8 and K7, respectively) were compatible with 9 out of 10 strains. Complex mating systems have been described for the genus *Alexandrium*.

Groups		(A)			(B)		(C)			(D)	(E)
	Strains	Q09	AY3	VALV2	DAVIS10	A4	ESTER2	AY2	ACC07	CERES8	K7
(A)	Q09	0	0	0	4	3	0	0	0	4	3
	AY3	0	0	0	2	2	0	0	0	2	2
	VALV2	0	0	0	1	4	0	0	0	3	1
(B)	DAVIS10	4	2	1	0	0	0	0	0	1	1
	A4	3	2	4	0	0	0	0	0	1	2
(C)	ESTER2	0	0	0	0	0	0	0	0	1	1
	AY2	0	0	0	0	0	0	0	0	1	1
	ACC07	0	0	0	0	0	0	0	0	1	1
(D)	CERES8	4	2	3	1	1	1	1	1	0	1
(E)	K7	3	2	1	1	2	1	1	1	1	0

Fig. 2. *Alexandrium catenella* mating groups. Shaded areas containing 5 groups A, B, C , D and E. Scoring criteria for cyst production: 0 = unsuccessful crosses; 1 to 4 correspond to <20, 21-100, 101-200 and >200 cysts ml^{-1}, respectively.

For example, a multiple mating system for *A. tamarense* in strains from eastern Canada (Destombe & Cembella 1990) and a homothallic system in *A. affine* from Californian waters (Band-Schmidt et al. 2003). It is therefore perhaps not surprising to find a more complex system in the Chilean *A. catenella* by experimenting with numerous clones, which certainly would enhance the likelihood of mating success in this highly variable ecosystem.

Overall reproductive compatibility of each *A. catenella* strain measured by compatibility index (CIs), average vigour (AVs) and reproductive compatibility (RCs) are summarized in Table 1. There were significant differences in response among the strains (ANOVA, $p<0.05$); however, the RCs index was clearly linked to the proximity of geographical origin of the isolates. An example is observed for strains within the group C, all from the southern Aysén region (Fig. 1), which presented the lowest reproductive compatibility indices among the clones. In contrast, LSU-rDNA and rDNA-ITS sequences were identical among the 10 strains, clustering only with one of the two distinct group I rDNA-ITS sequence types in Aguilera-Belmonte et al., (2011) (Fig.4). Therefore, no correlation between rDNA-ITS sequences and mating types was observed in this study.

Table 1. Reproductive compatibility (RCs), average vigour (AVs) and compatibility index (CIs) of ten Chilean *A. catenella* strains (arranged from north to south) after pairwise crosses among all strains.

Strain	CIs	AVs	RCs
Q09	0.50	3.40	1.70
CERES8	1.10	1.55	1.70
AY3	0.50	1.80	0.90
VALV2	0.50	2.00	1.00
DAVIS10	0.50	1.80	0.90
ESTER2	0.20	1.00	0.20
AY2	0.20	1.00	0.20
ACC07	0.30	1.00	0.30
A4	0.60	2.17	1.30
K7	1.10	1.36	1.50

Cyst production was highly variable among the strain inter-crossing experiments, ranging from 7±1.3 to 316.9±20.5 cysts ml^{-1} (using 400 cell ml^{-1} as inoculum). Cyst production rates (cyst d^{-1}) are presented in Fig. 3. Cyst formation started 26 days and finished 45 days after inoculation. Both, starting and ending periods were surprisingly synchronized. The highest cyst production rates were reached around 5-7 days after inoculation.

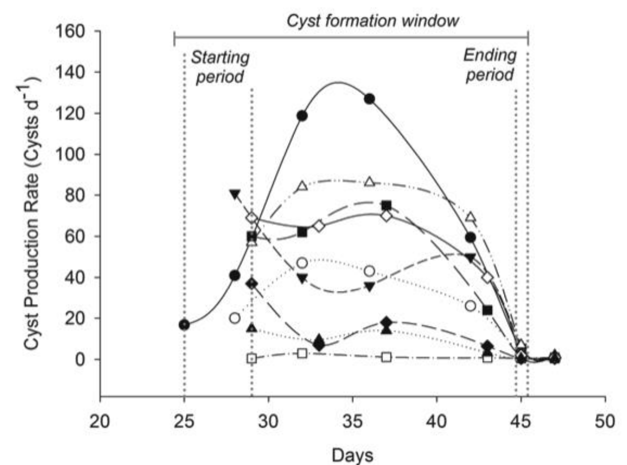

Fig. 3. Cyst production rate among Chilean *A. catenella* strains from nine pairwise crosses.

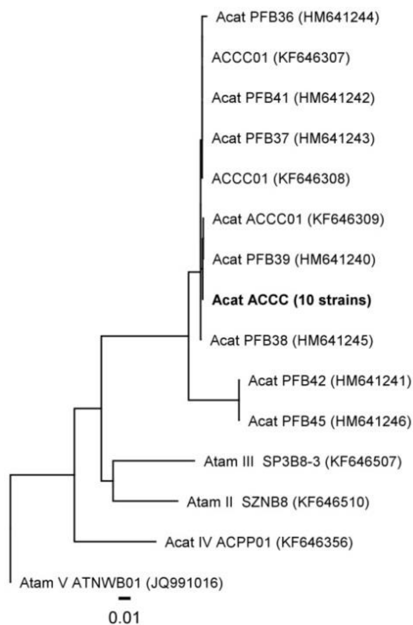

Fig. 4. Relationship of Chilean *A. catenella* rDNA-ITS sequences from this study (In bold: Acat ACCC) to those of Aguilera-Belmonte et al. (2011).

The successful production of cysts presented here suggests that low concentrations of cysts detected in sediments samples in the fjords, at least in the northern area, cannot be explained by this biological factor and might instead be the result of advection due to local circulation patterns as described by Silva et al. (1995), with a net flux occurring toward off shore waters between 0-50 m depth. In order to allow for more conclusive interpretation, further oceanographic studies focused on local water circulation and the use of sediment cyst traps must be pursued.

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Alexandrium cyst distribution and germination in Puget Sound WA, USA

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Abstract

The Puget Sound *Alexandrium* Harmful Algal Bloom (PS-AHAB - http://www.tiny.cc/psahab) program investigated cyst dynamics of the toxic dinoflagellate in the genus *Alexandrium*. This included mapping overwintering surface sediment cysts at 99 stations throughout Puget Sound in 2011, 2012 and 2013. The distribution patterns of cyst abundances were similar for all three years, but cyst concentrations generally decreased over time. The highest cyst concentrations were found north in Bellingham Bay, west of the Main Basin in Port Madison, Liberty Bay and Port Orchard and centrally in Quartermaster Harbor. Compared to a 2005 survey, the Bellingham Bay "seed bed" is new, whereas Quartermaster Harbor cyst concentrations have decreased by an order of magnitude. A monthly times series in Quartermaster Harbor from 2012-2013 found cyst abundances to vary by a factor of ~6 seasonally over the course of a year. In a related study, surface sediment cysts at thirty 2012 PS-AHAB stations were evaluated for their germination potential with results ranging from 16% to 66% viability. These results indicate that cyst abundances are relatively stable over a winter season, but winter cyst abundances and viability must be taken into account in order to determine the potential for toxic *Alexandrium* blooms the following season.

Keywords: *Alexandrium*, cysts, sediments, germination, Puget Sound

Introduction

Puget Sound, in the Pacific Northwest (PNW) region of the United States, is a complex estuarine system of interconnected deep fjords and shallow embayments linked to the Pacific Ocean through the Strait of Juan de Fuca. Shellfish harvest closures due to paralytic shellfish toxins (PSTs) in Puget Sound have increased in recent decades (Trainer *et al.* 2003), even though PSTs have been present in the PNW for centuries (Quayle 1969). PSTs in Puget Sound are produced by the dinoflagellate *Alexandrium*, which spends part of its lifecycle as a cyst in the sediment before germinating to form vegetative cells in the water column. These cells can then be ingested via filter feeding by bivalves, which concentrates these powerful neurotoxins, making the shellfish deadly to mammals if consumed. Toxic blooms of *Alexandrium* pose a challenge to the management of shellfish resources and human health in Puget Sound.

The species of *Alexandrium* thought to be responsible for the production of these toxins in Puget Sound has historically been identified as *Alexandrium catenella* (Whedon & Kofoid) Balech. This is synonymous with *Alexandrium tamarense* Group I, a provisional species name proposed by Lilly *et al.* (2007). However, the name *Alexandrium fundyense* has recently been proposed to replace all Group I strains of the *A. tamarense* species complex that includes *A. catenella* (John *et al.,* 2014a; 2014b). In light of the recent work by John *et al.* (2014a) and recognizing alternative recommendations by Wang *et al.* (2014), we will refer here only to the genus name *Alexandrium*. The Puget Sound *Alexandrium* Harmful Algal Bloom (PS-AHAB: *www.tiny.cc/psahab*) program, funded by NOAA/ECOHAB and Washington Sea Grant, seeks to understand environmental controls on the benthic (cyst) and planktonic life stages of the toxic dinoflagellate *Alexandrium*, and evaluate the effects of climate change on the timing and location of blooms in Puget Sound. Part of this study consisted of mapping interannual variations

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in *Alexandrium* cyst distribution in Puget Sound and conducting laboratory experiments to quantify the rates and timing of cyst germination related to exogenous and endogenous factors. In addition, *Alexandrium* cyst viability was determined throughout Puget Sound in surface sediments and changes in surface sediment cyst distributions were monitored in one bay over the course of a year to determine the effectiveness of winter cyst mapping as a tool to predict *Alexandrium* summer bloom potential. Results from the 2011, 2012 and 2013 spatial surveys of *Alexandrium* cyst distribution in the surface sediments of Puget Sound are presented here and compared to an earlier surface sediment cyst survey from 2005 (Horner *et al.* 2011). The time series of monthly surface sediment cyst abundances from 2012-2013 in Quartermaster Harbor is also presented. The results from the 2012 cyst germination viability experiment were presented at the 15th ICHA conference (Greengrove *et al*. 2014) and this paper is an update to that work. Details of other project experiments designed to investigate environmental and endogenous controls on cyst germination are presented in a separate paper (Moore *et al.* 2015).

Material and Methods

Field Sampling & Cyst Enumeration

Surface sediment *Alexandrium* cyst distribution mapping surveys were completed during winter in 2011, 2012 and 2013. Surveys consisted of 99 stations throughout all of Puget Sound, the Strait of Juan de Fuca and the San Juan Islands (Fig. 1). Sediment samples from the upper 0-1 cm and 1-3 cm were collected using a Craib corer (Anderson *et al.* 2003). Sediments were processed for cyst enumeration using the method of Yamaguchi *et al.* (1995), total organic content (loss-on-ignition) and grain size using a particle size analyzer. Monthly surface sediments were also sampled at two locations in Quartermaster Harbor (station 78 central bay and station 79 inner bay) from February 2012 through January 2013 using the same collection and processing procedure described above.

Results and Discussion

In 2011, the highest cyst concentrations were found in Bellingham Bay in the north, Port Madison, Liberty Bay and Port Orchard on the west side of the Main Basin and Quartermaster Harbor in central Puget Sound (Fig. 1). The pattern of Puget Sound cyst abundances remained the same for 2012 and 2013, but the concentrations were generally reduced over time (Fig. 1). Patterns of Puget Sound *Alexandrium* cyst abundances in surface sediment for the three surveys conducted as part of this study were compared with a survey conducted by another ECOHAB funded study in 2005 (Horner *et al.,* 2011) (Fig. 1). High cyst abundances were found in Quartermaster Harbor and Sequim Bay in 2005, consistent with historic PST hotspots (Trainer *et al*. 2003, Moore *et al*. 2009). A new seedbed area in Bellingham Bay was identified in this study that was not detected in 2005. The highest cyst concentration measured occurred at the Quartermaster Harbor seed bed in 2005. Cyst germination viability from selected stations all across Puget Sound ranged between 16-66% with no apparent difference between the surface 0-1cm and 1-3cm layers (Greengrove *et al*. 2014).

The monthly time series of cyst abundances at two locations in Quartermaster Harbor from 2012-2013 found cyst abundances to vary by a factor of ~6 with the lowest cyst abundances occurring in the spring (Apr) bloom season and the highest cyst abundances occurring in late fall (Oct/Nov) (Fig. 2). Vegetative cells of *Alexandrium* were found in the water column starting in April with the largest abundance of cells occurring between late June and early November, consistent with the decrease of cysts in the surface sediments (Moore *et al*. 2015). The presence of high vegetative cell concentrations led to shellfish bed closures in the bay during this bloom period (J. Borchert, Washington State Department of Health). Quartermaster Harbor is frequently closed to shellfish harvest during this time of year due to high PST levels (J. Borchert, Washington State Department of Health). These results indicate that cyst abundances are relatively stable over a winter season, but winter cyst abundances and viability must be taken into account in order to determine the potential for toxic *Alexandrium* blooms the following season.

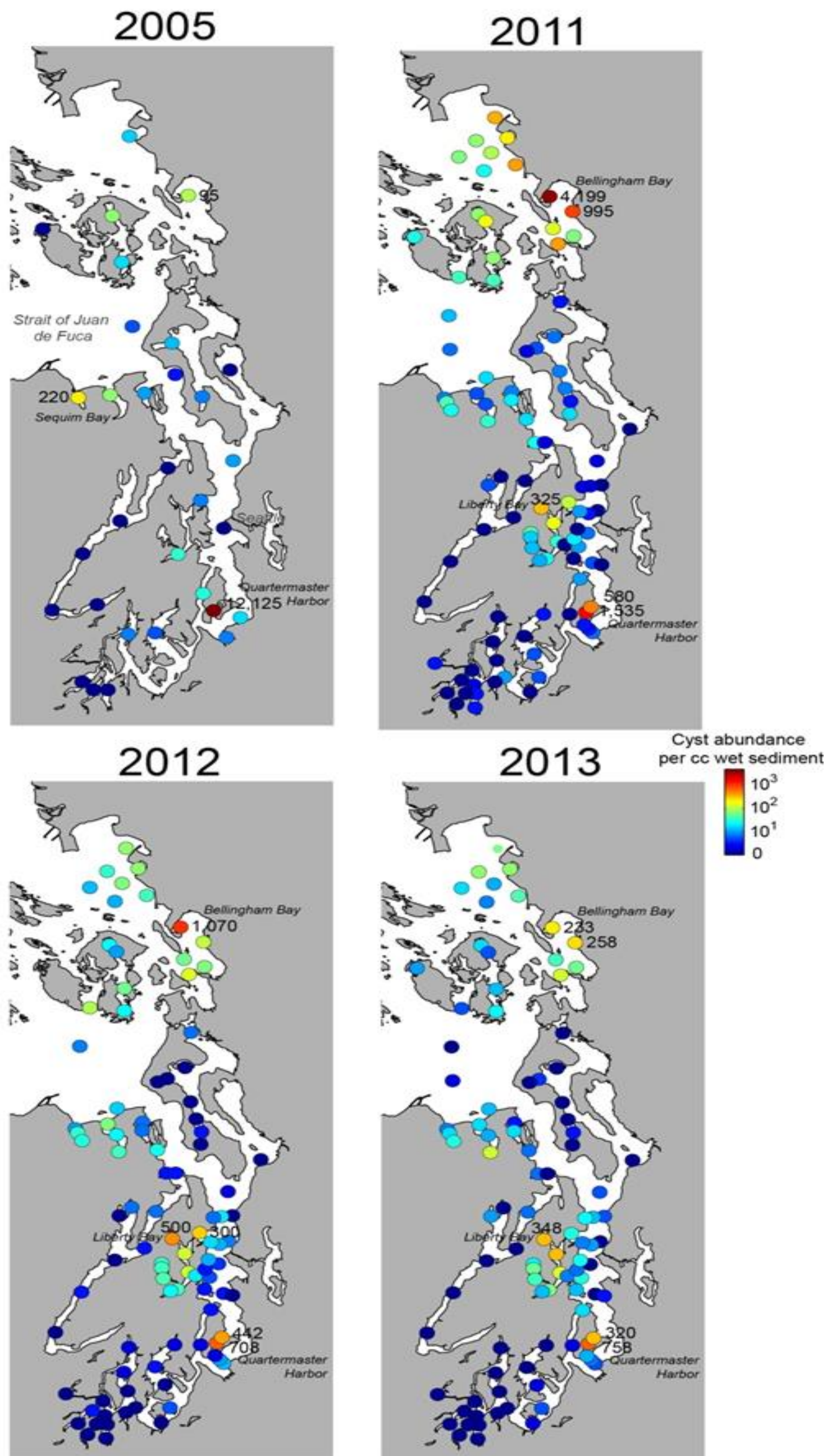

Fig. 1. Winter *Alexandrium* cyst maps for 2005, 2011, 2012 and 2013. The 2005 survey was conducted as part of another ECOHAB funded study (Horner *et al*., 2011)

The preliminary maps from the annual cyst distribution surveys were shared with Puget Sound health officials, marine resource managers and shellfish growers in the spring of each year, as part of the PS-AHAB "just-in-time" information dissemination program to stakeholders and all results were presented to stakeholders at a final workshop.

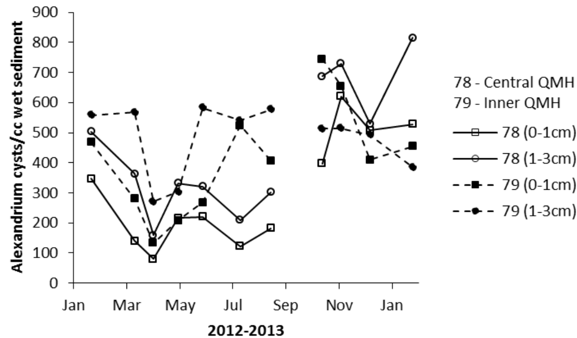

Fig. 2. Monthly *Alexandrium* cyst abundances per cc of wet sediment from 2012-2013 in Quartermaster Harbor at two locations, station 78 (solid line) in the central bay and station 79 (dashed line) in the inner bay. The squares are 0-1 cm and circles are 1-3 cm cyst abundances.

Laboratory experiments investigating the rates and timing of cyst germination related to exogenous and endogenous factors are presented in a separate paper (Moore *et al*. 2015). The improved understanding of the processes that govern cyst germination and bloom initiation provided by this study contribute towards the development of a predictive capacity for *Alexandrium* blooms in Puget Sound. Future work includes: laboratory experiments to determine the mandatory dormancy of *Alexandrium* cysts in Puget Sound and to more clearly determine whether or not an endogenous clock plays a role in excystment of these cysts in this region. Also, continued cyst mapping surveys are needed, as three years are not sufficient to confidently assess relationships between cyst abundances and the following season's shellfish toxicity values. Additional modeling work is needed to conduct particle tracking experiments and compare oceanographic transport pathways to the locations of seed beds and patterns of shellfish toxicity.

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A *Trichodesmium* bloom in south eastern Arabian Sea (SEAS): Observation of diatom-diazotrophic cyanobacterial associations in the bloom event

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Abstract

This study was undertaken with the objective of determining the distribution, diversity and ecological role of nitrogen fixing cyanobacteria and heterotrophic bacteria in the South Eastern Arabian Sea (SEAS: 9-13°N, 73-77°E). Sampling was carried out within the study area for a period of one year (March 2012 to February 2013). The water column was analyzed for physico-chemical variables, nutrient profiles and the distribution of nitrogen fixing heterotrophs and cyanobacteria. *Trichodesmium erythraeum* was found to be an important nitrogen fixing cyanobacteria in the SEAS forming blooms during spring inter-monsoon and winter monsoon periods. *T. erythraeum* blooms had densities of 1.84 x 10^6 filaments/L during the spring inter-monsoon period and 3.34 x 10^5 filaments/L during the winter monsoon period.A symbiotic diatom-diazotrophic-cyanobacteria(DDA) association between *Rhizosolenia hebetata* and *Rhizosolenia formosa* and the cyanobacteria *Richelia intracellularis* was documented for the first time during bloom of *T.erythraeum*. Our observations highlight the significance of DDAs and *Trichodesmium erythraeum* on the nutrient and energy budgets of phytoplankton in the oligotrophic environments of the Arabian Sea during the spring inter-monsoon.

Keywords: Trichodesmium; symbiosis; *Rhizosolenia-Richelia;* Southeastern Arabian Sea

Introduction

Oceanic nitrogen fixation by cyanobacteria play a vital role in providing fixed "New" nitrogen to the surface water communities (Karl *et al.,* 2002).The availability of fixed nitrogen (such as nitrate and ammonia) can limit the productivity of our seas (White, 2012). A unique group of open ocean diazotrophs, are the heterocystous cyanobacteria that live in symbiotic associations with other phytoplankton, primarily diatoms. The diatom-diazotroph associations (DDAs) are widely reported in oligotrophic waters and have the capacity to form episodic, largely monospecific blooms that exhibit very high rates of carbon and nitrogen fixation worldwide (Foster *et al.,*2007). It is estimated that *Trichodesmium* contributes 1 - 5 mmol N m^{-2} d^{-1} and diazotrophic diatoms, 0.4 - 2.4 mmol N m^{-2} $d^{-1}$of fixed nitrogen to the world's oceans (Poulton *et al.,* 2009). Globally, DDA's fix 4.79 Tg N y^{-1} (Carpenter *et al*., 1999) which forms almost 25% of total input of nitrogen to the sea (Poulton *et al.,* 2009).
One of the most conspicuous free-living diazotrophic species is the colony-forming cyanobacterium, *Trichodesmium* that is found throughout tropical and subtropical oceans and forms large-scale surface blooms (Capone *et al.,* 2005). The cyanobacteria, *Richelia intracellularis* and *Calothrix rhizosoleniae* were found in association with diatoms such as *Rhizosolenia* spp., *Hemiaulus* sp., *Bacteriastrum* sp., *Chaetoceros* sp. and as epiphyte or endosybiont in *Guinardia cylindrus*in warm tropical and subtropical oligotrophic waters (Gomez *et al*. 2005). Only a few workers have reported *Rhizosolenia-Richelia* associations from Indian waters. Iyengar and Desikachary (1944), Subrahmanyan (1946) and Kulkarni *et al*., (2010) reported the occurrence of this species from the southeast coast and there has been only one report (Padmakumar *et al.,* 2010) from the North-eastern Arabian Sea (NEAS).

Materials and Methods

This study was a part of seasonal observations on diversity and distribution of cyanobacteria and nitrogen fixing heterotrophic bacteria made in the south-eastern Arabian Sea (SEAS) along the Kochi and Mangalore transects during 2012. Surface samples were collected from four stations

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(of 13,30,50 and 100 m depth) from both transects from the *FORV Sagar Sampada*. A surface sample was also collected from a bloom of *Trichodesmium erythraeum* at a station off Kochi (9°57'52" N; 75°50'38" E).

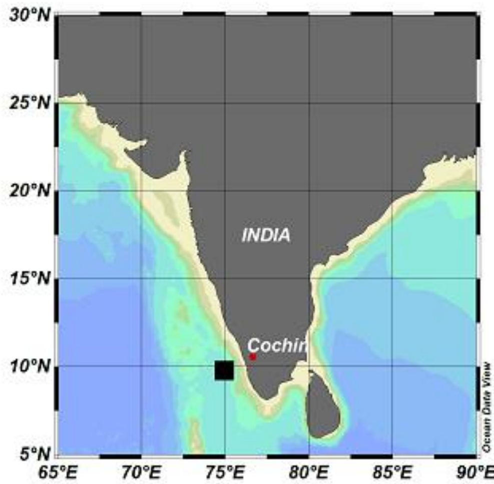

Fig.1. Map showing the location - *Richelia intracellularis* were symbiotic with *Rhizosolenia hebitata*

Temperature and salinity data were obtained with a CTD rosette system (Sea-Bird SBE 911 plus). Dissolved oxygen (Winkler's method) and nutrients in the sea water were estimated following Grasshoff *et al*. (1983). A bucket was used to collect sea surface water samples. Twenty five litres of each sample was filtered through 20 µm bolting silk net and preserved in 3% formalin and Lugol's Iodine.Samples were examined undera Nikon Eclipse microscope attached with Nikon DN 100 series digital camera and photomicrographs of the samples were taken. Identification of species was done using standard references (Tomas *et al*., 1997).

Results and Discussion

The hydrographic conditions of the study area are given in the Table 1. The *Trichodesmium erythraeum* bloom had a density of 1.84 x 10^6 filaments/L during spring inter-monsoon and 3.34 x 10^5 filaments/L during winter monsoon at the 50 m deep Kochi 50 m station. We observed the occurrence of the *Rhizosolenia-Richelia* (diatom-diazotrophic) association in the coastal waters of SEAS (Lat. 09° 57′52″ N and Long. 75° 50′ 38″ E) during April 2012 along with a bloom of *Trichodesmium erythraeum*.Analysis of collected samples using epifluorescence microscopy revealed there were two trichomes of *R. intracellaris* of unequal length within the cells of *Rhizosolenia hebetata.*
R. intracellularis Schmidt has been suggested to be among the most important and widespread nitrogen fixing endosymbiotic cyanobacteria in marine pelagic tropical and subtropical waters (Lyimo, 2011). It was found within five species of *Rhizosolenia*, *Hemiaulus membranaceus,H. hauckii, Guinardia cylindrus* and rarely as epiphyte to *Chaetoceros* spp. or freely in waters (Lyimo, 2011).

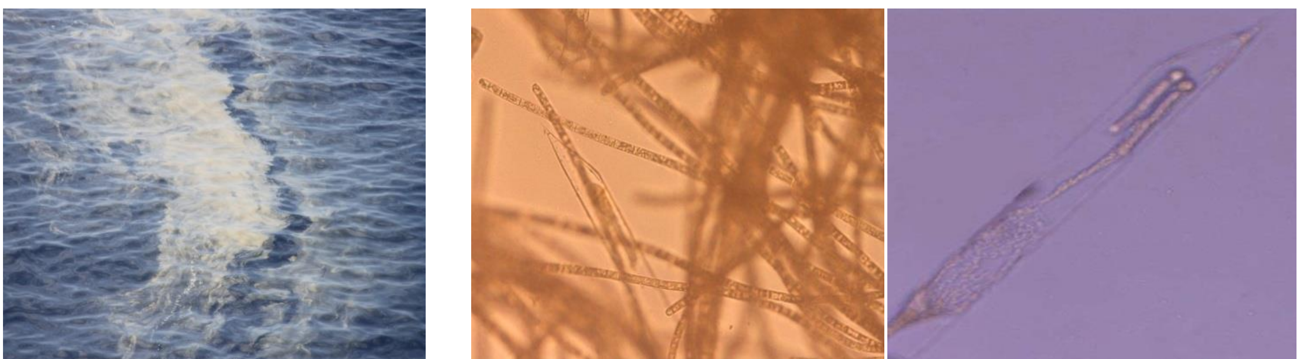

Fig. 2. (a) *T. erythraeum*bloom along the south eastern Arabian sea (b) *Rhizosolenia hebetata* with trichome of *R. intracellularis* among the filaments of *T. erythraeum*(x 400) (c) *R. hebetata* with trichome of *R. intracellularis*

Table 1. Hydrographical variables observed along the Kochi and mangalore transects during April 2012.

Variables	Range of observed values	
	Kochi	Mangalore
Temperature (°C)	30.1-30.7	30.1-31.4
Salinity (psu)	33.9-35.0	34.6-35.2
Wind Speed (ms^{-1})	2.4-13.9	2.4-9.9
DO (mlL^{-1})	4.0-4.9	4.1-4.7
Ammonia (µM)	0.41-1.9	0.4-1.7
Nitrate (µM)	0.06-1.9	0.04-0.24
Silicate (µM)	0.5-1.6	1.7-5.3
Phosphate (µM)	0.11-0.52	0.03-0.56

Trichodesmium erythraeum constituted 45-86% and 40-60% of total cyanobacteria on the Kochi and Mangalore transects respectively. *Rhizosolenia* sp. contributed to 0.4% and 0.6% of the total diatoms at the two transects respectively.

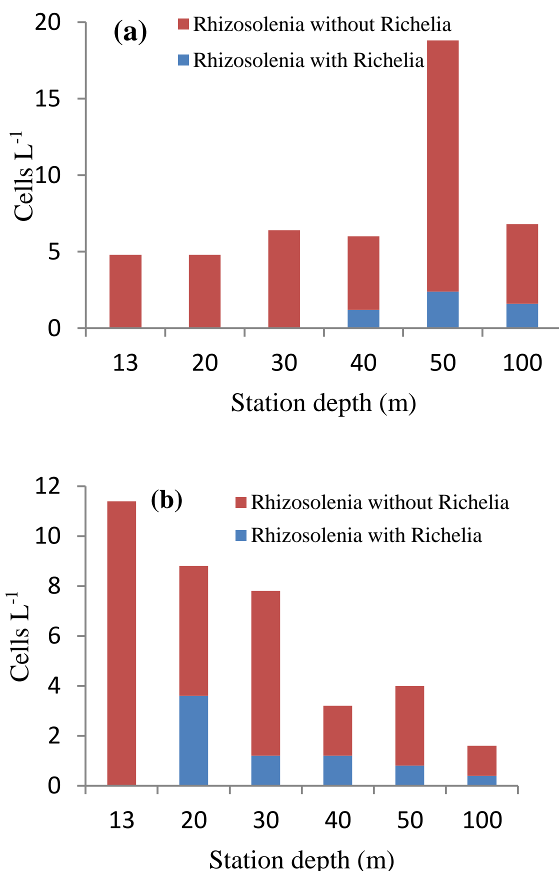

Fig. 3.(a) Cell density of *Rhizosolenia* with and without *Richelia intracellularis* at Kochi transect (b) Cell density of *Rhizosolenia* with and without *Richelia intracellularis* at Manglore transect.

The numerical abundance of diazotrophs was less than 5 cells L^{-1} on both the transects, suggesting they were in an initial phase of their occurrence. They were more widespread off Mangalore where they were observed at all stations except at station 1. *Rhizosolenia hebatata* with endosymbiotic *R. intracellularis* was observed at a density of 6 cells L^{-1}(Fig. 3) during spring inter monsoon. The bloom sample observed in the deeper stations off Kochi farther waters was dominated by *Trichodesmium erythraeum* (over 85%) while diatoms constituted only 12% (Fig.4). The nitrogen fixation rate of this *Trichodesmium* sp. bloom patches along with DDA contribute 3.6 nmol N/l/hr during spring Inter monsoon (Bhavya *et al*., 2015).

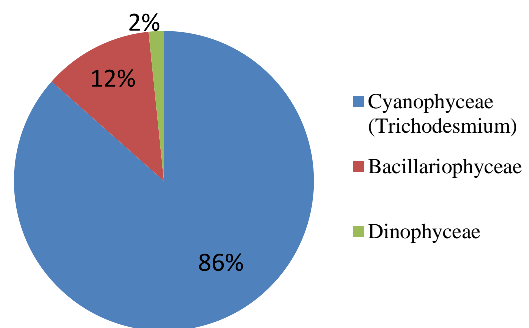

Fig. 4. Phytoplankton densities of bloom station during Spring intermonsoon season

Trichodesmium and diatom-diazotrophic symbiotic associations contribute significantly to fixing of new nitrogen into the SEAS. Oligotrophic conditions in the study area seem to trigger formation of the *Rhizosolenia – Richelia* symbiosis, which could be considered as an ecological adaptation to life in the oligotrophic ocean. Although there have been observations of blooms of *Trichodesmium*in the Arabian Sea, reports on the N_2 fixation by cyanobacteria to the marine nitrogen budget are relatively few (Westberry *et al*., 2006). According to Gandhi *et al*. (2011), N_2 fixation by *Trichodesmium* occurs mainly in the upper 10 m of ocean surface and their fluxes to the Arabian Sea were estimated at 15.4±1.5 Tg N y^{-1}, which is equivalent to ~92% of total 'new' nitrogen supply to the Arabian Sea and ~11% of the global N_2 fixation. During the study period, diatom diazotrophic associations were found in lower numbers in the SEAS. However, they symbiotically support non-cyanophyceans (diatoms) in fixing nitrogen for their metabolic activity, thereby playing a significant role in the nitrogen cycle of this region.

Conclusion

The present study highlights the presence of diatom-diazotroph symbiotic associations, which along with *Trichodesmium erythraeum* influence the nutrient and energy budgets of phytoplankton in the oligotrophic environment of the SEAS.

Acknowledgments

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Micro- and nano-fluidics around HAB cells

The *RheFFO** Working Group

(*Rheology, micro/nanoFluidics and bioFouling in the Oceans)

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Abstract

Have you ever wondered how algae stay so clean? Most flowering-plant leaves also stay clean. Under air, films of water and "dirt" are repelled. Repulsion forces the water into droplets that easily roll off because these leaves are covered in hydrophobic nanometre (nm) to micrometre (µm) sized grooves and pillars, producing superhydrophobicity (SH) at the surface. Similarly, most algal cells bear a glycocalyx of organic fibrils that give surface structure, and are often hydrophobic. Glycocalyxes serve many functions, but whether they produce SH is poorly known. SH coatings are being developed to prevent fouling of ships and aquaculture structures without using toxins, so this technology could help understand how algae defeat fouling. Glycocalyxes are composed of exopolymeric secretions (EPS), and algae sometimes make the water more viscous using this tightly and more loosely bound EPS. EPS is also sometimes sticky. SH cuticles on copepods may change ambient fluid microdynamics by allowing slip at their surfaces, and facilitate filter feeding. By managing ambient viscosity and surface properties including slipping and sticking, algae may have the tools to engineer ambient fluidics and stay clean and unfouled.

Keywords: Micro-algae, glycocalyx, fluidics, cell surface hydrophobicity, copepods

Bioengineering of viscosity and local flow by plankton

Previous studies have shown that the viscosity of ocean water is composed of a Newtonian component due to water and salt plus a non-Newtonian one due mainly to phytoplankton as well as bacteria. Such changes have been measured at mm to cm scales (Jenkinson and Sun, 2010; Seuront *et al.,* 2010). The exceptionally large biomasses and surface areas associated with cells and associated exopolymeric substances (EPS) in some HABs may give HAB species more potential , through quorum action, for management (bioengineering) of physical oceanographic and dispersion processes (Jenkinson and Wyatt, 1995), and may also provide an environment where they are easier to measure *in situ*.

Micro- and nanoscale fluidics around HAB and other algal cells are important in models of secretion and uptake (Mitchell *et al.*, 1985, 2013; Lazier and Mann, 1989). By managing of ambient viscosity as well as surface properties such as slipping and sticking, algae may have the tools to engineer ambient fluidics, distributions of processes involving substances such as nutrients and allelopathic substances, and stay clean and free of fouling. The remainder of this communication concerns possible bioengineering of processes only nm to mm from plankton cell surfaces.

A. Lincoln MacKenzie [Ed]. *Marine and Freshwater Harmful Algae 2014*. Proceedings of the 16th International Conference on Harmful. Algae. Cawthron Institute, Nelson, New Zealand and the International Society for the Study of Harmful Algae (ISSHA)

Wall slip

The notion of wall-slip, slipping of a sheared fluid at a wall (Fig. 1), was introduced by Navier (1823). Yet for over a century engineering manuals and physics textbooks taught "no-slip" as a quasi-universal premise for practical models of hydrodynamics (Jenkinson 2014). In the last 10-20 years, with the surge in machines, such as lab-on-a-chip, that incorporate microcapillary flow the idea of "no-slip" between liquid and solid surfaces has once again come to be regarded as "no more than a convenient approximation" (Rothstein 2010).

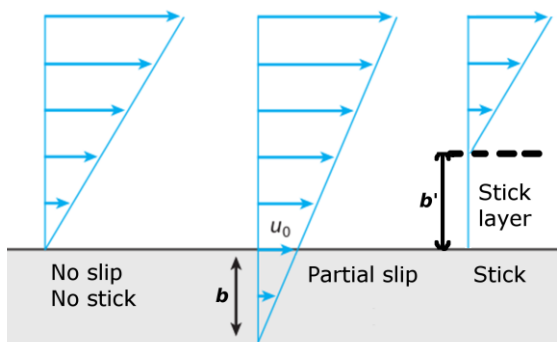

Fig. 1. Diagram of slipping and sticking and fluid-shearing at a solid interface. b is slip length; b' is sticking length; u_0 is slip velocity. b can range from nanometres to micrometres, or occasionally millimetres. Modified from Rothstein (2010).

Fig. 2. Lotus (water lily) leaf showing repelled water and "dirt". Size of photo ~10 cm. Photo: Ian Jenkinson.

The purity of the sacred lotus and plankton: slip layers, self-cleaning and anti-fouling,

Lotus leaves remain "pure", or dry and clean, because their leaf surfaces are superhydrophobic (SH) and therefore repel water and "dirt" (Fig. 2) (Barthlott and Neinhuis 1997; Thielicke, 2015). Hydrophobic surfaces can be SH when, in addition, they bear nano- or micro-sculpturing (Rothstein, 2010; Koch *et al.* 2009). On many terrestrial plant leaves, SH is produced by a wax-covered surface in the form of grooves or pillars (Fig 3). This principle has recently been incorporated into products such as: non-stick frying pans; dirt-repelling household wiping cloths; "green" (i.e. non-toxic) anti-biofouling, slippery surfaces for ships and structures used in aquaculture.

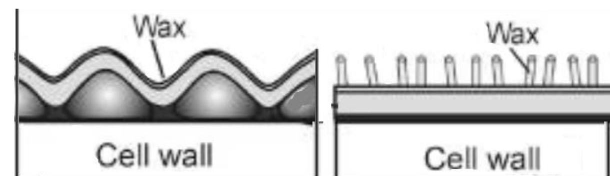

Fig. 3. Sketch of the origin of SH on leaves of flowering plants. Left – leaf with grooves; Right – micropillars. Adapted from Koch *et al*. (2009).

Anti-biofouling surfaces

Like the self-cleaning lotus leaves, many aquatic algae remain remarkably clean of "dirt", as well as of fouling organisms. While secretion by algae of mucus can reduce fouling (Boney 1981), the surfaces of many clean algae appear devoid of mucus. At µm scale many phytoplankton cells bear sculptured surfaces either when bare (Figs. 4, 5) or when covered with exopolymeric secretions (EPS) (Fig. 6), whether tightly bound as a glycocalyx or more loosely bound. Most algal and bacterial cells bear a glycocalyx of organic fibrils that give surface structure, and are often hydrophobic. Glycocalyxes serve many functions, but whether they produce SH is poorly known.

Associated with the progressive banning of toxic antifouling paints, there is much research into producing long-lasting anti-fouling and anti-corrosion SH coatings (Yang *et al.* 2015). Research in this field is benefiting from mimicking the surfaces of organisms. In return, research on fouling and antifouling by plankton can be inspired by such industrial research. The potential exists for collaboration between researchers on industrial antifouling coatings and those working on both fouling and antifouling by plankton organisms.

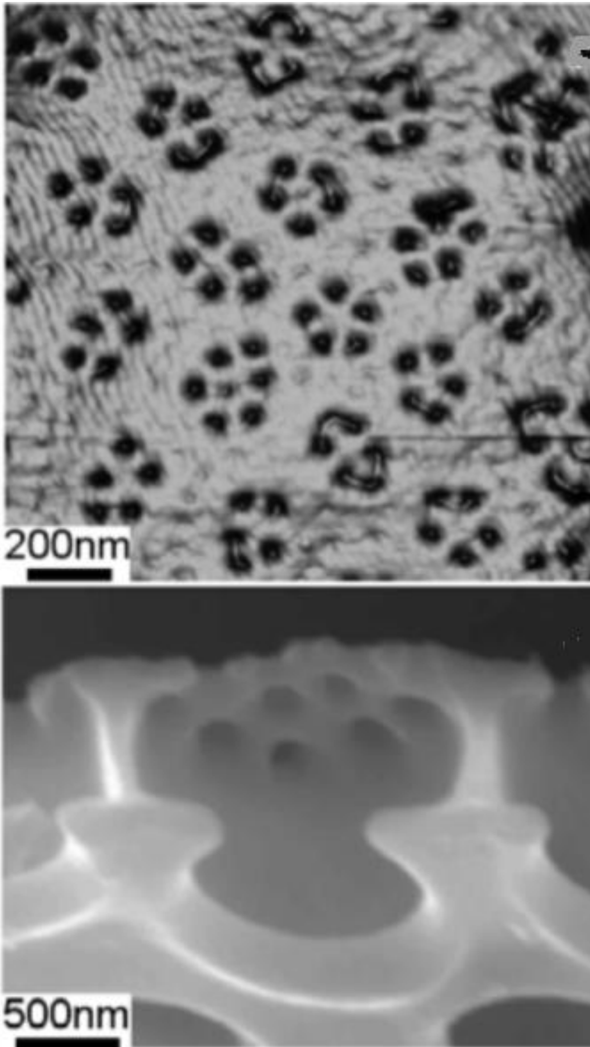

Fig. 4. Scanning electron micrograph (SEM) of detail of diatom valve Upper – *Coscinodiscus* sp.; lower – *Thalassiosira eccentrica*. Modified from Mitchell (2013).

Slippery liquid-infused porous surfaces (SLIPS)

SLIPS are SH surfaces in which the interstices between the sculptures (c.f. Fig. 3) are filled with a fluid, different from the overlying one, that is either locked in place, or constantly replaced, e.g. by secretion through pores, or both (Wong *et al.* 2011). SLIPS can be omniphobic, that is repellent to both hydrophobic and hydrophilic objects and are self-cleaning. In many phyla of harmful and other algae, the potential seems available for renewal of such fluid through pores between sculpturing. It should be investigated whether some harmful algae and protists may bear SLIPS that could modulate hydrodynamics and biofouling.

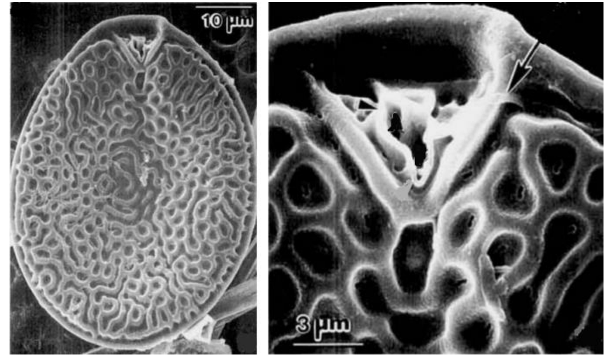

Fig. 5. SEM of the dinoflagellate *Prorocentrum reticulatum*, 55-60 x 40-45 µm. Note sculpture at different scales. Arrow (right) shows a bacterium. Detail from Faust (1997).

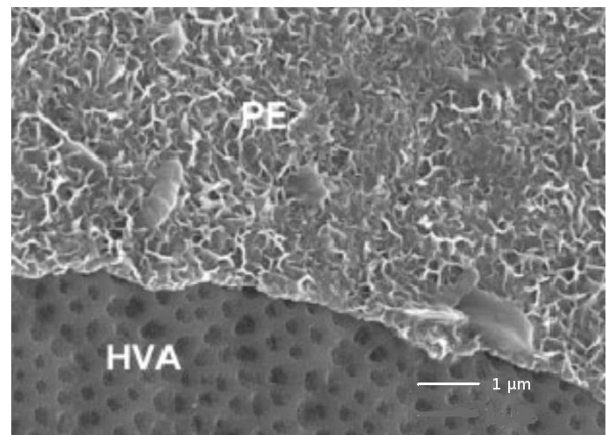

Fig. 6. SEM of *Coscinodiscus perforatus* cell to show hypovalve areolation (HVA) and overlying perifrustular envelope (PE). Reworked detail from Beninger and Decottignies (2009).

Copepods as predators of algae: their surfaces

Copepods are important in controlling HAB and other algae. However, HAB species may benefit from being able to defeat such predation. Like lotus leaves, copepods are believed to have waxy cuticles, often with surface sculpturing. Copepod feeding appendages (Fig. 7) bear two rows of setules typically 2 to 10 µm apart (Marshall and Orr, 1966). Whether these setules are used to filter phytoplankton is much debated, as observations of feeding, that apparently included filter feeding (Paffenhöfer *et al.* 1982) have been challenged by hydrodynamic models (Jørgensen 1983) with which it has been difficult to reconcile observations. The models have all used the no-slip assumption, however, new observations using better optics and higher-speed video have led to the total absence of filter-feeding being re-questioned (Kiørboe 2011). A slip length at the surface of setules of 1 µm or more may allow hydrodynamic models to be reconciled with filter

feeding, which needs to be investigated, with and without viscosity changed by more-or-less sticky algal mucus.

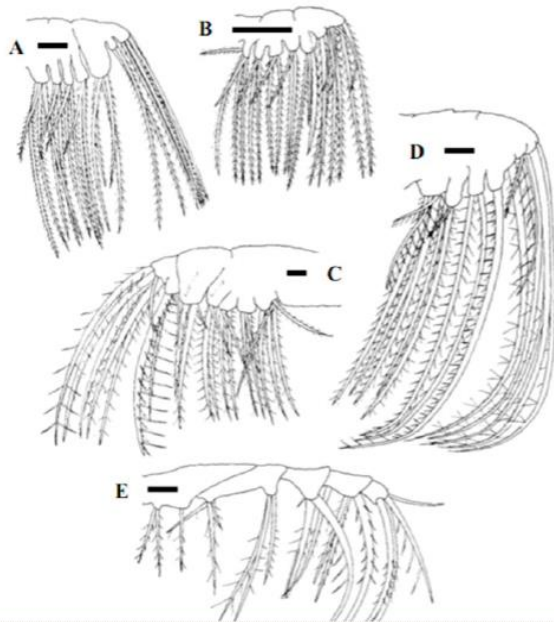

Fig. 7. Copepod feeding appendages, to show setae and setules. A – *Pseudocalanus*; B- *Temora*; C – *Centropages*; D – *Acartia*; E – *Oithona*. Scale bars – 20 µm. Modified from Marshall and Orr (1966). Reproduced with kind permission.

The *RheFFO Working Group*

RheFFO stands for Rheology, micro/nanoFluidics and bioFouling in the Oceans. The aim of the working group (WG) is to associate a corpus of researchers in the physical and biological ocean sciences, rheology, surface science and fouling/antifouling research, development and innovation. Harmful algae are a key part of it. It brings together science in modelling the environment with innovation of commercial products. Interested scientists may ask to join, or just associate, with this WG.

Acknowledgements

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This is publication No. 1 of the *RheFFO* Working Group.

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The rise of harmful algal blooms in Abu Dhabi coastal waters - the potential role of eutrophication and climate change

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Abstract

The increasing frequency of harmful algal blooms (HABs) and subsequent environmental impacts are a rising threat to the Arabian Gulf's coastal resources, economy and public health. Eutrophication and climate change are two processes that may promote HABs in Abu Dhabi waters. To understand the role of these two processes, an intensive marine water quality monitoring program was initiated in 2002, and 12 years of data reveal a significant annual increase in harmful algal bloom incidents. The changing patterns of phytoplankton taxa and the frequency of bloom formation are correlated to eutrophication and regional climate change, in particular changes in temperature and salinity. Increasing nutrient discharges exacerbate eutrophication. Increased nutrient concentrations significantly exceeding background levels and the resultant blooms were associated with widespread harmful impacts including hypoxic events (0.20mg/L dissolved oxygen), fish kills (*Nematalosa nasus*) and subsequent benthic organism losses. Extensive dredging and land reclamation activities could also be exacerbating the situation. Due to extreme climatic conditions the Abu Dhabi Emirate is highly vulnerable to the effects of regional climate change. Hence apart from eutrophication, the role of climate change in phytoplankton genera and harmful algal bloom expansion will be discussed in detail. In addition, the paper also covers HAB prevention, control and mitigation efforts.

Keywords: Harmful algal blooms, Nutrients, Eutrophication, Climate change

Introduction

Marine harmful algal blooms (HAB) are a natural phenomenon throughout the world and represent a significant threat to marine ecosystems and human health. The increasing frequency of HABs and subsequent environmental impacts pose a compelling and rising risk to the Arabian Gulf's coastal resources, economy and public health. Over the past several years the numbers of harmful algal bloom incidents in Abu Dhabi waters have increased along with urban and coastal development. There are various mechanisms that increase the frequency of HABs (Yin 2003). In heavily polluted waters nutrients are the main drivers (Paerl 1997). Eutrophication favors periodic proliferation and dominance of harmful blooms, both in planktonic and benthic (Paerl *et al.* 2001, 2008) environments. Rabalais *et al.* (2009) pointed out some important mechanisms by which global change may affect eutrophication of coastal ecosystems. The objective of the present paper is to identify the role of eutrophication and climate change in the increase in HABs in Abu Dhabi waters.

Materials and Methods

Environment Agency-Abu Dhabi (EAD) has conducted regular, systematic phytoplankton surveys for surveillance, identification, enumeration, and study the ecology of HABs in the Emirate's territorial waters since 2002, starting with 8 sites and increasing to 20 sites in 2011. Phytoplankton samples were collected using 20 micron bolting silk net (Hydro-bios). Filtrates were preserved with Lugol's solution. Phytoplankton samples were also collected from bloom areas and non-bloom patches. Quantitative and qualitative analyses of phytoplankton were carried out by using a Sedgewick Rafter counting cell under a Nikon Inverted microscope following standard identification keys. The area of the bloom, mortality of fishes (if any), was estimated on the basis of visual observation. Water quality parameters such as salinity, temperature, pH, dissolved oxygen and chlorophyll-a were recorded. Principal nutrients (nitrate-N, phosphate-P, ammonia-N and silicate-Si) were quantified using standard methods. In situ algal bloom observations and chronological HAB

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events were compiled to illustrate the trend of HAB events from 2002 onwards. Shamal (strong northwesterly winds) and rainfall data (Windguru, 2014) were integrated with the analysis of climate change and algal blooms.

Results

Data collected over the past 12 years reveal that surface sea water temperature has increased

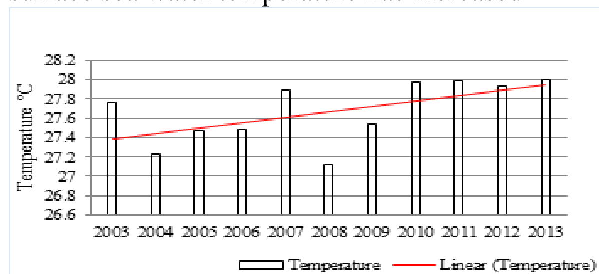

Fig 1. Temperature-annual variation (mean values)

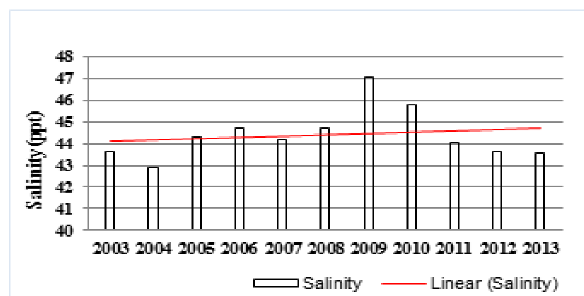

Fig 2. Salinity -annual variation (mean values)

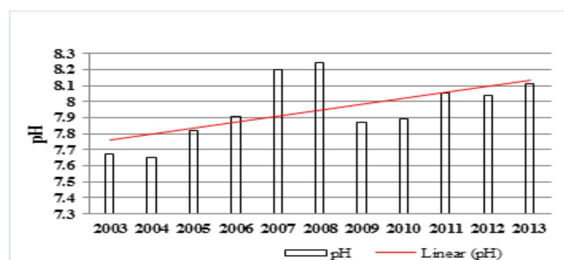

Fig 3. pH- annual variation (mean values)

slightly, with values fluctuating between 18.4°C and 34.9°C (Fig.1). Variation in salinity values were unremarkable in Abu Dhabi, pH was slightly elevated, fluctuating between 7.4 and 8.5.

Nutrient levels increased over the study period, particularly nitrate, phosphate and silicate; whereas ammonia levels trended downwards (Fig. 4 & 5). Along with temperature, chlorophyll concentrations in Abu Dhabi waters rose (Fig. 6). Regarding phytoplankton, the present study revealed that diatoms appear in greatest abundance, followed by dinoflagellates. However, an increase in the number of dinoflagellates over the past few decades has been observed in Arabian Gulf. Along with the number of species, the frequency of phytoplankton blooms has also increased in Abu Dhabi waters (Fig. 7).

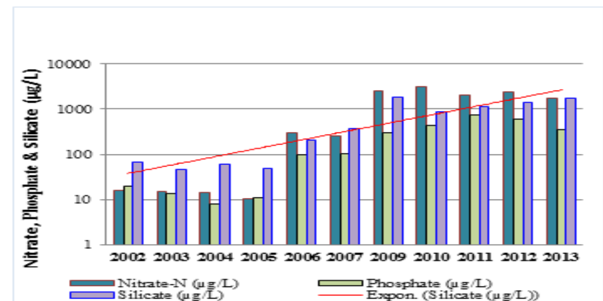

Fig 4. Variation of NO_3, PO_4, & SO_4-annual mean values

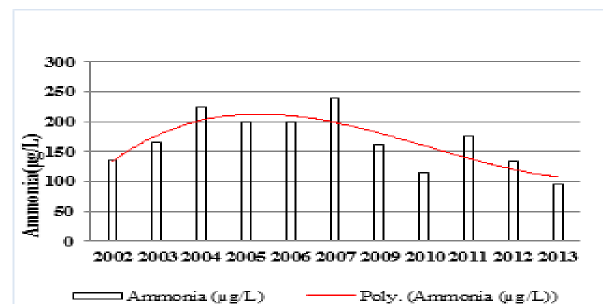

Fig 5. Variation of ammonia –annual mean values

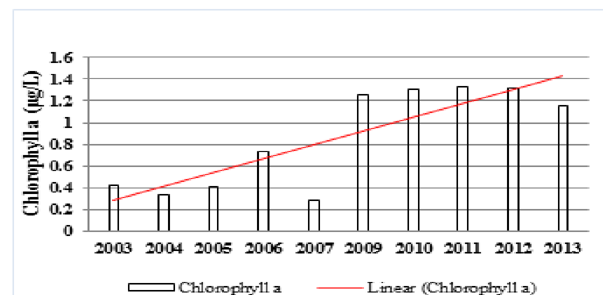

Fig 6. Variation of chlorophyll-a annual mean values

Discussion

HAB are an increasingly common phenomenon in coastal waters worldwide, and nutrient enrichment appears to promote these events (Anderson *et al.* 2008). In Abu Dhabi waters nutrient over-enrichment due to economic activity is considered a prime promoter of HABs. The number of algal blooms has increased every year for the past 12 years, reaching a maximum in 2012 with 29 incidents. In general, nutrients are introduced to coastal waters through human and animal wastes, industrial effluents, atmospheric deposition, and non-point source runoff of fertilizers. Factors

specific to Abu Dhabi Emirate may also include isolated raw sewage treatment overflows, discharge of treated wastewater into marine waters, and seasonal wind and rain events. Recent studies have begun to investigate the hypothesis that nutrients occurring in dust may be deposited in the UAE's coastal waters as a result of strong Shamal winds (Hamza *et al.* 2011). Rainfall and sandstorm frequencies increased over the past several years (Fig. 8). These winds may be depositing nutrients such as iron and nitrogen, which help fuel phytoplankton productivity, but further research is needed to determine the extent to which dust storms may play a role in localized eutrophication.

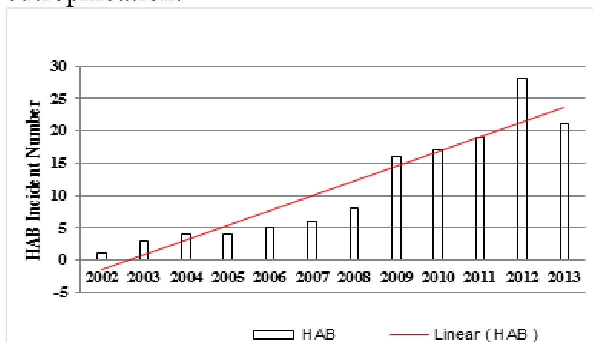

Fig-7. HAB incidents during different years

Sources contributing to eutrophication were investigated. Various outfalls were identified, categorized and monitored continuously for illegal discharges. Population growth has resulted in natural landscape alterations, including residential construction in coastal areas. Nutrient loads also increased due to infrastructure development in offshore islands. Groundwater drainage systems developed along the coast became sources of nutrient discharges. Another category, point sources, includes industrial and municipal wastewater treatment plants, storm water outfalls, and combined sewer overflows. The other category of nutrient loading is nonpoint sources which include watershed runoff and atmospheric deposition. The principal source of nutrients in Abu Dhabi City is nitrogen inputs from sewage treatment plants, with at least 400,000 m3/day of secondary treated sewage water being discharged into Mussafah South Channel.

The increase in primary production and algal blooms results in elevated organic matter production. As oxygen consumption begins to exceed replenishment, anoxia and even hypoxia begin to develop. The bottom waters are the first area of the system to experience hypoxia, decreasing the benthos community (Paerl 2005). In the Abu Dhabi area a highly eutrophic channel has been hypoxic for the past several years.

Algal blooms diminishing light levels in the marine ecosystem affects seagrass. Since seagrass is a critical habitat, their loss loosens sediment on the sea floor, adding to particulate suspension and turbidity, further blocking light penetration. The loss of food and oxygen encourages mobile marine organisms to migrate and immobile species die (Rabalais 2002). As the habitat becomes less desirable, smaller species are dominated by predators, (Rabalais, 2002), shifting the food web and decreasing biodiversity. The function and structure of the phytoplankton community continues to change with the addition of HABs to regular blooms and also reduce dissolved oxygen concentrations.

Our results also indicate that phytoplankton community composition may be altered by changes in N, P, and Si ratios (Schollhorn and Graneli 1993). As has been observed in the Baltic and North Seas, a change in community structure shows an increase in flagellates. In general, global climate change will likely result in higher water temperatures, stronger stratification, and increased inflows of freshwater and nutrients to coastal waters in many areas of the world (Rost 2008).

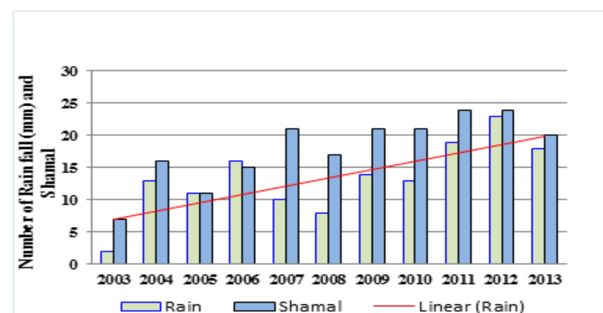

Fig 8. Annual mean rainfall and total number of Shamals

Analysis of existing data collected since 2002 in Abu Dhabi waters show that changes in some water parameters demonstrated a strong relationship with climate change. Current data (Fig. 1) collected over several years, shows that surface water temperature in Abu Dhabi waters is increasing. Concurrently the chlorophyll-a concentrations are trending upwards. In addition the study revealed that diatoms dominated, followed by dinoflagellates. However, an increase in the number of dinoflagellates in the Arabian Gulf has been observed over the past few decades. In general, as atmospheric carbon dioxide

increases, it is absorbed into ocean water, making it more acidic. However in Abu Dhabi waters pH is generally increasing (Fig 3). In Abu Dhabi waters the salinity variation during the past several years were insignificant. The Arabian Gulf region has experienced a slight increase in the frequency of cyclones and rainfall. These increased the frequency of algal bloom incidences, especially in Abu Dhabi waters. Dust storms/sandstorms are common in the Arabian Gulf region. Dust events are most likely when the wind is off the desert to the south, considerably less so when the wind is from the northwest with shamal conditions, in which dust has to be transported a significant distance across the Arabian Gulf (Hamza *et. al.* 2011). Climate change increases the frequency of sandstorms and shamals. The sandstorms deposit copious amounts of nutrients in the sea, resulting in increasing phytoplankton productivity and bloom incidences. Chlorophyll-a concentrations in Abu Dhabi waters have increased. Monitoring data and case studies provide preliminary evidence of climate change-induced effects on phytoplankton concentrations. More studies are needed, including long term monitoring of marine phytoplankton in the coastal waters to provide scientifically sound information to the public and decision makers, as well as to monitor the effects of mitigation measures and management plans.

Conclusions

Data collected over the past 12 years suggests a strong correlation between eutrophication and HABs, and a lesser but observable connection between climate change and increased incidence of HABs. Eutrophication and climate change not only influence harmful algae, but also lead to cumulative impacts. Furthermore, climate change itself exacerbates eutrophication in many ways (EPA-USA 2013). Given the significant knowledge gaps regarding climate change's effects on the marine ecosystems of the Arabian Gulf, proactive steps are needed to expand knowledge, cooperation, and implementation of mitigation and adaptation policies.

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Ciguatera

Gambierdiscus and *Ostreopsis* from New Zealand, the Kermadec Islands and the Cook Islands and the risk of ciguatera fish poisoning in New Zealand

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Abstract

Gambierdiscus cf. *yasumotoi* identified from Northland, New Zealand, in 2013 was found to produce putative MTX-3, and *O. siamensis* isolates, which produced palytoxin-like compounds, formed dense epiphytic blooms at that time. Artificial substrate samplers were deployed during a 2014 sampling trip to enable comparative assessment of the epiphytic dinoflagellates against standard methods, and fish were analysed for palytoxins. Again, *O. siamensis* was a major toxic bloom former and *G.* cf. *yasumotoi* was present at low levels. The Kermadec Islands group, a New Zealand territory, was visited in 2013 by the research vessel Braveheart (led by staff of Auckland Museum) and *Ostreopsis* and *Gambierdiscus* were isolated from samples collected from Raoul Island; an *Ostreopsis* isolate proved to be an undescribed species. *Gambierdiscus polynesiensis*, *G. australes, G. pacificus* and *O. ovata* were isolated from the Cook Islands in 2013. *G. polynesiensis* produced CTX and *G. australes* produced MTX and all species produced putative MTX-3. An isolate of *O. ovata* produced palytoxin-like compounds. The current CFP risk from New Zealand seafood is negligible, and people reported to have CFP symptoms in New Zealand have invariably been poisoned through eating contaminated reef fish while visiting the Pacific Islands. However, there is the potential for increased risk of CFP in New Zealand due to tropicalisation of northern coastal waters, and risk monitoring will continue.

Keywords: Gambierdiscus, *Ostreopsis*, ciguatoxin, maitotoxin, palytoxin, New Zealand, Kermadec Islands, Cook Islands

Introduction

The epiphytic dinoflagellate genus *Gambierdiscus* is associated with ciguatera fish poisoning (CFP), a potentially fatal illness. CFP is caused by consumption of fish that have accumulated ciguatoxins (CTX) and/or maitotoxins (MTX). These toxins are produced by species in the genus *Gambierdiscus.* Palytoxin-like compounds are produced by *Ostreopsis* species and may confound diagnoses of CFP. In order to further understand the risk from these epiphytic dinoflagellates, three sites were investigated, namely sub-tropical northern New Zealand, Raoul Island in the Kermadec Island group (a New Zealand territory) and Rarotonga, Cook Islands, which is a known 'hot spot' for CFP (Fig.1; Rongo and van Woesik 2011, 2012).

During 2013, *Gambierdiscus* cf. *yasumotoi* was found in Northland, New Zealand (Rhodes *et al.* 2014a) and a compressed form of *Gambierdiscus* was isolated from Boat Cove, Raoul Island, in the Kermadec Islands group, but did not survive in culture (Rhodes *et al.* 2014c). The Kermadec Islands lie 1,100 km northeast of New Zealand and include 747,000 ha of marine reserve. The islands are a stepping stone for fish migrations between New Zealand and more northern tropical waters, such as the Cook Islands. Several isolates of *G. polynesiensis*, *G. australes* and *G. pacificus* were isolated from the Cook Islands, where CFP is a common and sometimes lethal illness (Rhodes *et al.* 2014b); all produce toxins which are implicated in CFP.

The discovery of blooms of *G. carpenteri* in coastal waters of temperate New South Wales, Australia (Kohli *et al.* 2014a,b), raises concerns about the ability of the dinoflagellates to adapt to cooler waters. In any event, the likelihood of rising sea water temperatures raises the possibility of an increased risk of CFP as *Gambierdiscus* increases its geographic range (Llewellyn 2010). The risk of CFP occurring in New Zealand will therefore continue to be assessed and efforts made to further characterise *Gambierdiscus* and CFP in the Pacific region.

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Material and Methods

Collection, isolation and culturing
Dinoflagellates were isolated from samples collected in the Bay of Islands, Northland, New Zealand (Lat. 35°16'S, Long. 174°15'E), Boat Cove, Raoul Island, in the Kermadec Islands group (collected by SCUBA diving; Lat. 29°16'S, Long. 177°55'W) and Rarotonga, Cook Islands (Lat. 21°13'S; Long. 159°43'W and Lat. 21°15'S, Long. 159°48'W; Fig. 1). Isolation and culturing were carried out as described previously (Rhodes *et al.* 2014a,b,c).
Artificial substrate samplers (Tester *et al.* 2014) were deployed at three sites within Te Uenga Bay, Bay of Islands, New Zealand. The average cell concentrations from the three samplers were determined for each site, with controls of water and adjacent seaweeds sampled for comparison. Selected isolates are maintained in the Cawthron Institute Culture Collection of Micro-algae.
DNA sequence analyses
DNA sequencing data analyses were carried out as described previously (Rhodes *et al.* 2014 b).
Toxin analyses
Gambierdiscus and *Ostreopsis* cultures (approx. 200 ml; 10-20,000 cells per ml) were centrifuged (3000 *g*) and pellets extracted with methanol (analytical grade). CTX, MTX and MTX-3 analogues were analysed by LC-MS/MS as described previously (Rhodes *et al.* 2014c) and palytoxin-like compounds as described by Selwood *et al.* (2012).
Toxicity
Freeze-dried cell pellets from dinoflagellate batch cultures were extracted with ethanol, dried and resuspended in 1% Tween 60 in saline as described previously (Rhodes *et al.* 2014b), and administered by intraperitoneal (i.p.) injection or gavage to Swiss albino mice (18-20 g) at various dose levels. LD_{50} values were then determined (OECD 2006; all experiments were approved by the Institutional Animal Ethics Committee).

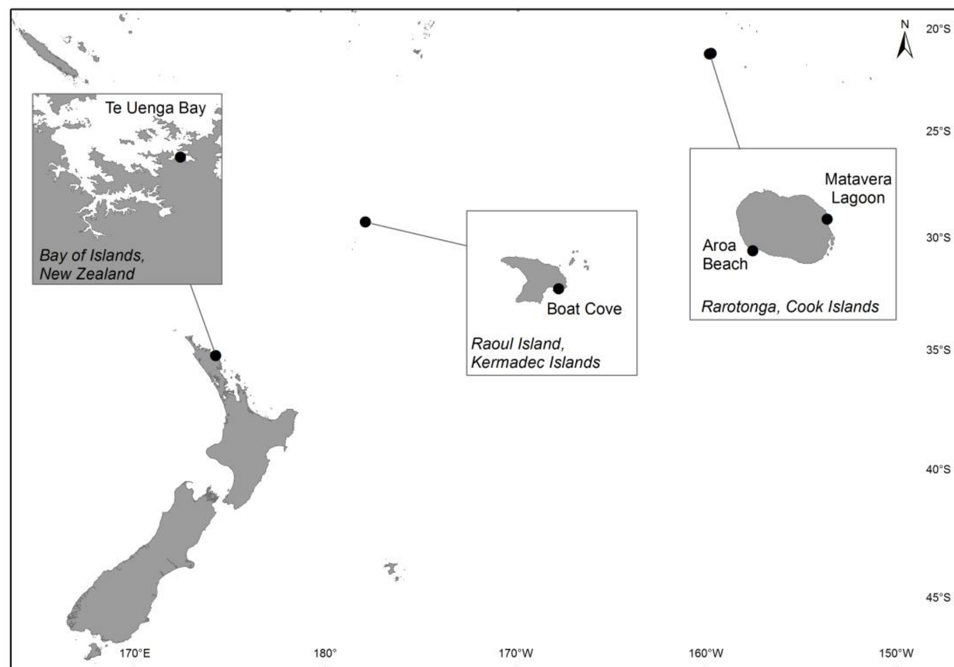

Fig. 1. Map to show sampling sites for isolation of *Gambierdiscus* and *Ostreopsis* in New Zealand, Kermadec Islands and the Cook Islands.

Results and Discussion

G. cf. *yasumotoi* was discovered at Te Uenga Bay, Northland, New Zealand, in 2013 on the turf-forming macroalga, *Jania rosea* (Rhodes *et al.* 2014a). An intensive sampling trip was undertaken in 2014 to investigate the area more rigorously. Sea surface temperatures (SST) at this site averaged 20-21°C. Several cells of *G.* cf. *yasumotoi* were observed under the light microscope in fixed (Lugol's iodine) samples collected at Te Uenga Bay using the artificial samplers (Tester *et al.* 2014). *G.* cf. *yasumotoi* was also detected in samples from Motuarohia Island , north of Russell (Lat. -35°13'57.36" Long.

174°10'0.12"; SST 21-22°C), as determined by metabarcoding analyses (Smith *et al.*, in prep.).

O. siamensis was a major bloom former in 2013. In 2014, *O. siamensis* cells attached to artificial samplers after 24 hrs at two sites (B and C) in Te Uenga Bay (Lat. -35°14'53.45"; Long. 174°14'24.62"), with average concentrations of 9000 and 22,500 cells/grid respectively; no cells attached at site A. This showed a markedly greater cell concentration than in the surrounding waters, which averaged 0-200 cells/L. Dense blooms of *O. siamensis* at other macroalgal rich sites sampled throughout the Bay of Islands, including Motuarohia Island, confirmed that *O. siamensis* was blooming epiphytically throughout the region. Isolates produced palytoxin-like compounds as determined by LC-MS/MS. Co-occurring dinoflagellate species included *Amphidinium thermaeum, Coolia malayensis, Gymnodinium dorsalisulcum, Prorocentrum lima, P. rhathymum,* and *P. triestinum*, while the toxic cyanobacteria genus *Oscillatoria* was abundant.

The concentrations of *Gambierdiscus* were approx. 10,000 times lower than those of *O. siamensis*, which suggests an extremely low risk of CFP from consuming fish in the Bay of Islands area. The high cell concentrations of *O. siamensis* suggested a potential risk of poisoning from palytoxin-like compounds, but herbivorous fish gut analyses were negative for palytoxins. Gastropods (*Lunella (=Turbo) smaragdus*, blue mussels (*Mytilus edulis*) and various hermit crabs, sponges and ascidians were also negative for palytoxin.

The voyage to Boat Cove, Raoul Island, resulted in the isolation, but sadly not the culture, of a compressed form of *Gambierdiscus*. An undescribed *Ostreopsis* species was successfully cultured (CAWD221). Other isolates included *C. malayensis, A. massartii, P. lima* and *P. emarginatum* (Rhodes *et al.* 2014c). Further information on *Gambierdiscus* species presence and toxin production will be sought at the next opportunity.

Isolates from the Cook Islands included *G. australes* (CAWD149, 216), *G. pacificus* (CAWD213, 227) and *G. polynesiensis* (CAWD212). CAWD149 produces MTX (8.3 pg/cell) and CAWD212 produces CTX (18.2 pg/cell); all isolates produce putative MTX-3 (Fig. 2; Rhodes et al. 2014b). Extracts of *G. polynesiensis* and *G. pacificus* were equally toxic to mice by i.p. injection, but the former was >30 times more toxic by gavage (LD_{50} 7.9 mg/kg v LD_{50} 251 mg/kg respectively; Rhodes *et al.* 2014b), demonstrating the importance of determining toxicity via the oral route.

Interestingly, *G. polynesiensis* caused spinal curvature in Zebra fish embryo tests (FET) with an EC_{50} of 0.398 mg/L, while MTX-3 (CAWD213 extracts) caused only a minor response. However, MTX plus MTX-3 (CAWD149) elicited an EC_{50} of 0.249 mg/L. The FET therefore shows promise as a functional assay and will be investigated further (refer Argyle *et al.*, this proceedings). There was no response to any *Ostreopsis* isolates.

In conclusion, *Gambierdiscus* and CFP are known throughout the Pacific region from tropical to temperate waters. *Gambierdiscus* grows in New Zealand's sub-tropical waters and there is therefore a risk of CFP in New Zealand in the future. Adaptation to cooler temperatures combined with the trend for tropicalisation of more temperate regions increases that risk. Currently there is minimal likelihood of CFP from fish caught in New Zealand's mainland waters, but there are anecdotal reports of CFP from consumption of fish from the Kermadec Islands region and also anecdotal reports of occasional tropical fish being caught in the Bay of Islands. In the Cook Islands, the incidence of CFP is high and the producers of CTX and MTX have been identified from Rarotongan lagoons.

Massive *Ostreopsis* blooms occur throughout the Pacific, including northern New Zealand, but so far no palytoxin-like compounds have been detected in New Zealand fish and there is low toxicity in mice via the oral route compared to i.p. injection, suggesting low uptake in humans (Munday 2014). However, palytoxin poisoning remains a potential risk and chemical tests have been developed to monitor for it should it be regulated (Selwood *et al.* 2012). Risk assessments of CFP and palytoxin poisoning in New Zealand will be on-going.

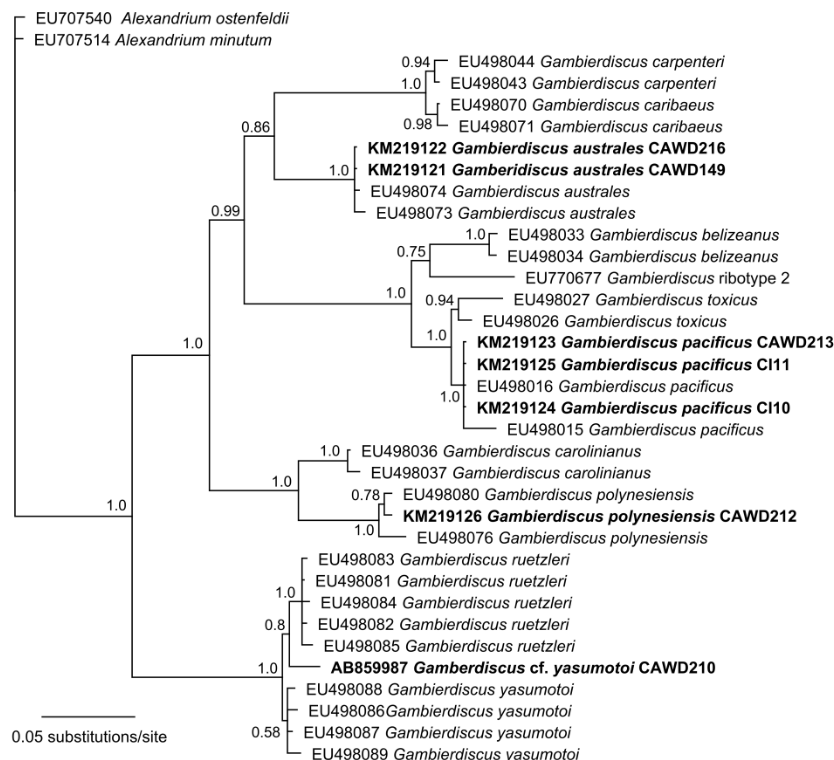

Fig. 2. Phylogenetic analysis of *Gambierdiscus* species from New Zealand (CAWD210) and the Cook Islands (isolates from this study in bold) showing alignment of partial LSU rDNA sequences in the D8-D10 region using Bayesian analyses. Values at nodes represent Bayesian posterior probability support. Scale bar: substitutions per site.

Acknowledgements

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Ciguatoxin concentrations in invasive lionfish estimated using a fluorescent receptor binding assay

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Abstract

Invasive lionfish have proliferated throughout the Caribbean, causing extensive environmental damage to reef ecosystems. Given the high densities and extensive range, reef managers are developing local lionfish fisheries as a control strategy. Lionfish have a mild flavor, firm white meat, freeze well, and command high market prices, all factors that favor the establishment of a fishery. The successes of this approach in the Caribbean and Gulf of Mexico will depend on the degree to which lionfish accumulate ciguatoxins (CTX) produced by co-occurring dinoflagellates in the genus *Gambierdiscus*. This is of concern because consumption of fish containing elevated CTX concentrations can result in ciguatera fish poisoning (CFP), the largest cause of non-bacterial seafood poisoning worldwide. In this study, CTX concentrations in lionfish were surveyed throughout the Caribbean and Gulf of Mexico using a novel fluorescent receptor binding assay (RBA_f). Approximately 11% of the lionfish contained CTX concentrations slightly above the safety level recommended by FDA, but significantly below levels documented to cause human illness. Implications for developing a lionfish fishery and the need to survey CTX levels in other reef fish commonly consumed without adverse health effects are discussed.

Keywords: Caribbean, ciguatera, invasive species, lionfish, *Pterois volitans/miles,* receptor binding assay

Introduction

Invasive Indo-Pacific lionfish (*Pterois volitans / miles*) have spread throughout the Caribbean, causing extensive impacts to reef fish communities (Morris 2012). The development of commercial fisheries to control lionfish populations and mitigate their adverse impacts may depend on the extent to which these fish bioaccumulate ciguatoxins (CTXs). In this study, we adapted the fluorescent receptor binding assays (RBA_f) developed for brevetoxins (PbTxs) (McCall *et al*. 2012, 2014) for detection of ciguatoxins in fish. This adaptation is possible because PbTxs and CTXs bind the same site on voltage-gated sodium channels. The assay was used to screen lionfish collected from around the Caribbean and Gulf of Mexico and the results were expressed as ppb C-CTX-1 equivalents.

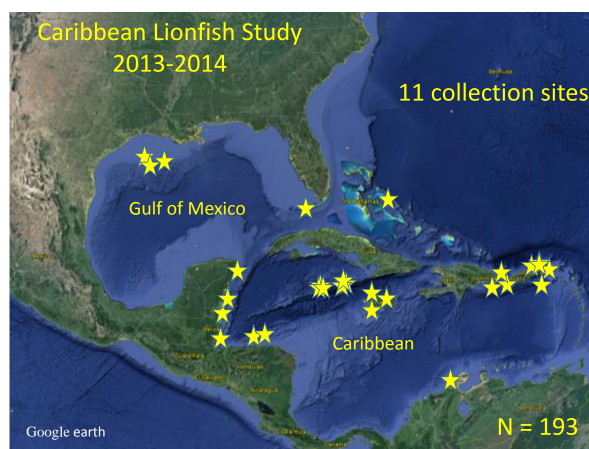

Fig. 1. Yellow stars indicate the origin of the lionfish samples analyzed in this study.

Material and Methods

Samples. Lionfish were collected from 11 locations (>30 sampling sites) from around the Caribbean and Gulf of Mexico by spearfishing or live capture in 2013 and 2014 (Fig. 1). The fish tested weighed from 150-1100 g and were collected at depths ranging from 5 - 47 m.

Tissue extraction. Tissue samples were extracted using a modified protocol developed by Darius *et al.* (2007). Briefly, whole fish or fillets were

thawed to room temperature and triplicate 5 g subsamples of flesh were removed from each specimen, placed in 50 mL conical tubes and incubated at 70°C for one hour. This step eliminated native fluorescence in the samples. Each tissue sample was then extracted with 7 mL of methanol and homogenized with a finger sonicator (Q-Sonica, Q700, Newtown, Connecticut) for 1 minute. The conical tubes were capped and placed in a water bath sonicator (Branson, 1800, Danbury, Connecticut) for two hours. Upon completion of sonication, the tissues were removed from the sonicator and incubated at room temperature overnight (14-16 hours). The extracts were then centrifuged at 4700 rpms for 5 minutes. The resulting supernatant was decanted into a 20 mL glass scintillation vial and adjusted to 70% methanol : 30% water (typically this only required adding ~ 3 mL of Milli-Q water). Each supernatant was then passed through a Waters Sep pak® Plus C_{18} solid phase extraction (SPE) column (WAT020515, 360 mg) preconditioned with 10 mL of 100% and 70% methanol : 30% water using a Supelco visiprep™ (St. Louis, Missouri) DL vacuum manifold. After extracts were loaded onto SPE columns, the columns were washed with 70% methanol : 30% water twice (7 mL x 2) and eluted with 7 mL of 90% methanol : 10% water into glass scintillation vials. Vials were then transferred to a nitrogen evaporator (Organomation Associates, Inc., N-EVAP 111, Berlin Massachusetts) and the extracts reduced to less than two mL under ultra-high purity nitrogen at 50 °C. The concentrated extracts were then transferred to two mL HPLC vials, reduced to dryness, sealed and stored at -20°C until analysis. Selected fish tissues were spiked with P-CTX-3C and processed to determine recovery efficiencies (85 ± 2%) and to confirm that the assay had a linear response over the 0.1-0.5 ppb P-CTX-3C equivalents range.

Synaptosome preparation. Synaptosomes rich in sodium channel receptors which serve as the target for the assay were prepared using a modification of the Poli *et al.* (1986) procedure as described in McCall *et al*. (2012).

Fluorescent receptor binding assay and standard curves. A fluorescence based receptor-binding assay (RBA_f) was used to prepare standard curves and determine toxicity in fish samples based on the protocol of McCall *et al*. (2012, 2014). Gilson Microman positive displacement pipettes (Gilson, Inc., Middleton, Wisconsin) were used for greater precision when pipetting solutions containing organic solvents.

P-CTX-3C (WAKO Chemicals, Richmond, Virginia), or C-CTX-1 (obtained from R. Lewis, University of Queensland) standards were made to a concentration of 1 ng μL^{-1} 200 proof ethanol. The standards were then diluted 1:10 and used to prepare a standard curve ranging from 10^{-8} to 10^{-13} g mL^{-1} of toxin.

The RBA_f assay buffer contained 50 mM HEPES, 130 mM choline chloride, 5.4 mM KCl, 1.7 mM $MgSO_4$, 5.5 mM glucose, 6.1 mM ethylene glycol, 1 g L^{-1} bovine serum albumin, 3 to 4 g L^{-1} Trizma® base (added until the pH was 7.4), 200 µL L^{-1} protease inhibitor cocktail (P-8340) and two drops of Tween-20 detergent (~0.02%). The buffer was stored at 4°C. All reagents were obtained from Sigma Aldrich (St. Louis, Missouri). The labeled PbTx conjugate (1 mM), which competes with for the synaptosome sodium channel sites, was dissolved in 100% methanol and stored at -20°C in the dark. Just prior to running an assay, 2 µL of BODIPY–PbTx-2 solution was added per 10 mL of buffer in low light and the mixture was vortexed for one minute.

Once the standards, assay buffer, BODIPY–PbTx-2, and extracted samples were prepared, the assay was assembled in a deep well polystyrene 96-well assay plate (PALL AcroPrep, Ann Arbor, Michigan) as follows. First, 200 µl of either diluted standard or sample extract was added to the appropriate wells. Next, 50 µL each of synaptosomes and BODIPY-PbTx-2 solution were added to each well using a multichannel pipettor. Finally, another 200 µL of buffer was added for a total assay volume of 500 µL per well. The combined reagents were allowed to incubate on ice in the dark (styrofoam cooler) while gently mixing (80 rpms) on a shaker (Thermo Scientific, Max Q 2000) for 2 hours to allow PbTx-2 or CTX standards to compete with the BODIPY-PbTx-2 for the synaptosomal sodium channel binding sites.

At the end of the incubation, Pall AcroPrep™ Advance 350 one μm glass fiber filter plates (96-well format) were placed on a Pall® multi-well plate manifold vacuum apparatus. A 250 µL aliquot of assay buffer was added to each well in the plate and then aspirated through the filter under vacuum (10 cm Hg). Under these

conditions, it took 10-15 seconds for the assay buffer to pass through the membrane at the bottom of each well. To ensure that the plate was fully dry, the vacuum was released; the plate was removed from the vacuum manifold tapped dry 3-4 times to remove any residual fluid retained on the side of the wells. The plate was then placed on the manifold and the process repeated twice. The plate was read using a FLUOstar Omega fluorometer (BMG Labtech, Germany) with a 505 nm long band pass dichroic, a 490-10 nm excitation filter and a 520-10 nm emission filter to obtain the relative fluorescence units (RFUs) for each well. This measurement was taken because initial studies indicated that there was a small 5% inter-well variation in the native fluorescence of the plates. Next, reaction mixtures prepared in the polystyrene 96-well plates were transferred to the buffer-washed filter plate mounted in the vacuum manifold as before. Once the samples were pulled through the filter plate, each well was rinsed with 250 μL of assay buffer. The plate was again vacuum filtered, blotted dry and read as previously described.

Once the RFU values for each standard curve or sample was determined, the background fluorescence for each well was subtracted to correct for the ~5% inter-well variability. Next, the lowest value RFU for the P-CTX-3C standard curve (highest toxin concentration) was subtracted from each well to account for any background fluorescence due to non-specific binding of the BODIPY– PbTx-2 to the synaptosomes or filter plate. The data were then scaled so that the corrected RFUs from the standard curve samples containing no P-CTX-3C represented 100% specific binding and the RFUs from the highest P-CTX-3C concentration was set to ~0% binding. The resulting normalized RFUs versus concentration data were used to estimate IC_{50} values using a non-linear regression curve-fit analysis program (GraphPad Prism version 4.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com).

Fish assays. The 5 g lionfish extracts were resuspended in 1000 µL of 1:9 methanol : water and the RBA_f assays carried out as described above. The only exception was that extracts were diluted 1:3 rather than 1:10 for the standards. The 1:3 dilution scheme was use because it produced more reliable IC_{50} estimates for the low or non-detectible levels of CTX found in the samples.

The resulting RFU (% binding) results were corrected for plate variation and non-specific fluorescence as described for the standard curve above. IC_{50} values (g fish tissue mL^{-1}) were determined and the P-CTX-3C equivalents (ng per g fish tissue, ppb) present in the fish were calculated by dividing the P-CTX-3C IC_{50} (ng mL^{-1}) by the extract IC_{50} (g fish tissue mL^{-1}) (Darius *et al.* 2007). Using the previously determined relationship between the P-CTX-3C and C-CTX-1 IC_{50} values it was possible to express the P-CTX-3C results as C-CTX-1 equivalents. Repeated spike experiments showed that reliable estimates between 0.1 and 0.5 ppb P-CTX-3C (= 0.074 - 0.37 C-CTX-1 equivalents) were possible.

The final data on C-CTX-1 equivalents in lionfish for each region were combined and displayed as pie charts showing what proportion of the fish had concentrations below the detection limit (<0.07 ppb C-CTX-1 eq.), detectible but below the recommended FDA safety level (0.07-0.1 ppb C-CTX-1 eq.), within 10X of the FDA safety level (0.1-1.0 C-CTX-1 eq.), and those with concentrations >1.0 ppb C-CTX-1 eq. (known to cause human illness).

Results and Discussion

This study represents the first Caribbean wide assessment of CTX levels in lionfish. Most of the fish examined contained toxin concentrations below the detection limit or were measurable but below the FDA recommended safety limit of 0.1 ppb C-CTX-1 equivalents. Approximately 11% of the fish (n = 193) assayed contained CTX concentrations above the FDA recommended safety limit of 0.1 ppb C-CTX-1 equivalents. Most of these fell in the 0.1 to 0.2 ppb C-CTX-1 range. The recommended 0.1 ppb safety limit was determined by screening meal remnants from documented CFP cases to establish the lowest CTX concentrations causing human illness (~1.0 ppb C-CTX-1 eq.), then dividing that value by 10 (Dickey and Plakas 2010). Robertson *et al.* (2014) similarly found that 12% of lionfish from the U.S. Virgin Islands fell within the 0.1 – 1.0 ppb C-CTX-1 range with most of the fish also exhibiting concentrations at the lower end of this range.

The highest frequency of lionfish containing measurable toxin in this study occurred in the central Caribbean, with the highest toxin values found in the eastern Caribbean (Fig. 2). This

toxicity pattern matches the region wide CFP incidence rates reported in Tester *et al*. (2010) and corresponds to the areas with the highest and most stable water temperatures. In contrast, some regions in the Gulf of Mexico and Caribbean report few or no instances of CFP, such as along the coast of Colombia (Tester *et al*. 2010). Lionfish obtained from several of these regions did not contain measurable CTX (Fig. 2), indicating that establishment of a lionfish fishery in these areas may pose little to no threat to human health.

In the central Caribbean, there are reports indicating localized "hot" spots where fish are frequently ciguatoxic. Whether lionfish in these areas can accumulate elevated concentrations of CTX approaching 1.0 ppb is unknown as no data exist on accumulation and depuration rates in this species. To date, however, there are no FDA reports of CFP from consumption of lionfish, despite these fish being routinely eaten throughout the region. Additional survey studies are needed to determine if the CTX levels in lionfish are any higher than in reef fish with similar feeding ecologies that are routinely consumed without adverse health effect. If the CTX levels are similar, then it would support the development of a broader lionfish fishery. If the levels in lionfish are higher, then a more regional approach to establishing and managing the fishery would be required. The data in this paper also support the use of the RBA_f assay, which can be completed in less than 2.5 h, as a screening tool to identify those fish whose CTX levels need to be secondarily confirmed using either N2a cytotoxicity or LC-MS assays.

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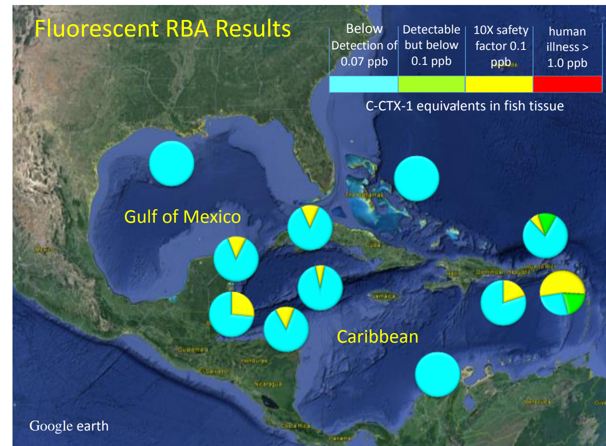

Fig. 2. Graphical presentation of the proportion of lionfish from each sampling location contain-ing below detection levels of CTX (light blue), detectable but below the FDA recommended CTX safety level (green), within the 10-fold safety factor zone (0.07-0.1 ppb, yellow), or concentrations known to cause human illness (>1.0 ppb, red).

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A practical approach for *Gambierdiscus* species monitoring in the Caribbean

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Abstract
Ciguatera fish poisoning (CFP) is the most common HAB-related illness in the world and is expected to increase in frequency in the Caribbean region as oceans warm. However, practical and methodological limitations prevent widespread monitoring for CFP toxins in seafood. Monitoring for toxic *Gambierdiscus* blooms, the origin of CFP toxins in the marine food web, is an alternative to mitigate CFP outbreaks. Two species-specific molecular detection tools show promise for monitoring *Gambierdiscus* cells, qPCR and FISH. The high throughput of qPCR and its ability to detect co-occurring *Gambierdiscus* species at very low abundances is more practical for routine monitoring when >2 species co-occur. FISH assays may be added to enhance the accuracy of cell concentrations for potential regulatory action during *Gambierdiscus* blooms. Used in concert with the artificial substrate method to quantify *Gambierdiscus* species, the molecular assays offer a two-tiered approach for abundance-based monitoring and CFP risk assessment.

Keywords: Ciguatoxin, Benthic harmful algal blooms (BHAB), Benthic dinoflagellate, Screen method

Introduction

HAB monitoring is conducted to provide advance warning of blooms such that adverse effects of toxins on residents and sensitive coastal ecosystems can be mitigated. There are two primary approaches—quantifying the abundance of toxic cells or measuring toxin concentrations in water or tissue samples. Currently, neither approach is being used systematically for ciguatera fish poisoning (CFP), an illness caused by bioconcentration of algal toxins (ciguatoxins, CTXs) in marine biota. CTXs are produced by benthic dinoflagellates in the genus *Gambierdiscus* and enter marine food webs primarily via ingestion of cells by herbivorous and surface-feeding fish and invertebrates (Bagnis *et al.* 1980, 1985, Chinain *et al*. 2010, GEOHAB 2012). In the Caribbean, CFP occurs most commonly in the islands in the northeastern region where temperatures are warmest and most stable (Tester *et al.* 2010). Widespread monitoring for CTXs in fish has been impractical because it requires complex chemical extractions and time consuming quantification methods hampered by lack of commercial toxin standards (Holland *et al*. 2013).

Cell-based monitoring for *Gambierdiscus* has also been problematic because of sample collection and replication issues. Traditionally, *Gambierdiscus* abundance has been assessed by collection of macrophytes or other natural substrates, followed by enumeration of associated *Gambierdiscus* cells by microscopy. However, *Gambierdiscus* abundance is often extremely patchy, requiring substantial replication to adequately assess cell concentrations (Lobel *et al.* 1988, Tester *et al*. 2014). Replication has posed difficulties including availability of the same substrate across space and time and covariation of *Gambierdiscus* abundance with that of substrates. These issues have led to the development of the artificial substrate (AS) method for assessing *Gambierdiscus* abundance, where units of substrate (fiberglass screen rectangles) are placed at monitoring sites, incubated for 24 h, and then retrieved and processed like natural substrates (Tester *et al*. 2014, Tester and Kibler, in press). Cell abundance is then expressed as cells cm^{-2} or cells 100 cm^{-2}. The AS method has proven highly effective for quantifying *Gambierdiscus* abundance in a variety of tropical and subtropical habitats, but the use of this technique for systematic monitoring is limited by the co-occurrence of multiple *Gambierdiscus* species.

Currently, there are at least 13 *Gambierdiscus* species, most of which are virtually indistinguishable via routine microscopy (Litaker *et al*. 2009, Fraga *et al*. 2011, Fraga and Rodrigues 2014). Of these, toxicity data are available for

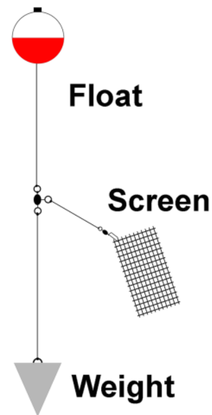

Fig. 1 Diagram of artificial substrate assembly, from Tester et al. (2014).

only *G. belizeanus* (120 fg P-CTX-3C Eq. $cell^{-1}$), *G caribaeus* (1-9 fg C-CTX-1 Eq. $cell^{-1}$) and *Gambierdiscus* ribotype 2 (31-54 fg C-CTX-1 Eq. $cell^{-1}$) (Lartigue *et al*. 2009, Chinain *et al.* 2010), although data for other *Gambierdiscus* species are now imminent (Hardison *et al*., in prep.). Because some species are 2-3 orders of magnitude more toxic than others, there is a critical need for a practical and cost effective monitoring method capable of rapidly differentiating the co-occurring *Gambierdiscus* species and quantifying their abundance before it reaches potentially dangerous levels. The subtle morphological features which distinguish *Gambierdiscus* species make molecular methods the only reliable options for identification and quantification. Here, we consider the application of two molecular techniques to monitoring *Gambierdiscus* abundance in tandem with the artificial substrate method as a mitigation tool for CFP in the Caribbean region.

Monitoring Method

Monitoring sites and sampling design—The first steps for monitoring are to determine where *Gambierdiscus* cells occur, typical levels of abundance, when their abundance is highest, and the species present. In the Caribbean, *Gambierdiscus* cells tend to be most abundant in shallow (<10 m), protected coral reef, seagrass and mangrove habitats, but cells occur at lower densities over much wider areas (Tindall and Morton 1998). If no prior data exist, the simplest way to characterize the *Gambierdiscus* assemblage is to conduct preliminary sampling using natural substrates. Samples of macrophytes and other materials are collected from a number of sites with ambient seawater. Associated *Gambierdiscus* cells are separated via shaking and sieving, and abundance is expressed per gram wet weight of substrate (Fig. 2; Litaker *et al*. 2010). The results of these surveys can serve as starting point to guide systematic monitoring with artificial substrates.

Artificial substrate (AS) method—The AS method was developed to assess *Gambierdiscus* abundance using any replicated sampling design without relying on availability of natural substrates. Detailed information about the AS method is given in Tester *et al*. (2014). Briefly, uniform rectangular pieces of fiberglass screen mesh, sized to fit the opening of 0.5-2.0 L wide mouth sample jars, are placed within ~20 cm of the bottom with a subsurface float (Fig. 1). Screens should be randomly distributed with sufficient replication to overcome the patchy distribution of *Gambierdiscus* cells (n = 2-10; Lobel *et al.* 1988, Watt 1998, Tester *et al.* 2014). After 24 h, screens are carefully collected in sample jars and shaken for 5-10 s to dislodge *Gambierdiscus* cells before sieving through 100-500 µm mesh to remove coarse sediment, detritus and other large particlulates (Fig. 2). The volume of the filtrate is recorded before one or more 100-1000 ml aliquots are homogenized and filtered through 47 mm diameter pieces of 20-30 µm pore size nylon mesh. The nylon mesh is then transferred to 50 ml test tubes for chemical preservation in 25 ml of fixative (see below) pending molecular analysis (Fig. 2). Biweekly sampling is sufficient during slow growth seasons, while weekly sampling is more appropriate during *Gambierdiscus* bloom seasons.

Molecular Analysis—Two species-specific molecular methods show potential for *Gambierdiscus* monitoring. The first, quantitative PCR (qPCR), uses species-specific primers to amplify DNA extracted from samples; the rate of DNA amplification is directly proportional to the number of cells present. Quantitative assays for *G. belizeanus*, *G. caribaeus*, *G. carolinianus*, *G. carpenteri* and *Gambierdiscus* ribotype 2 have already been developed (Vandersea *et al*. 2012). The second method, Fluoescent *in situ* hybridization (FISH), is a whole cell technique where fluorescently labeled, species-specific probes bind to the RNA in target cells, which are then counted via epifluorescent microscopy. FISH assays have been developed for *G. belizeanus*, *G.*

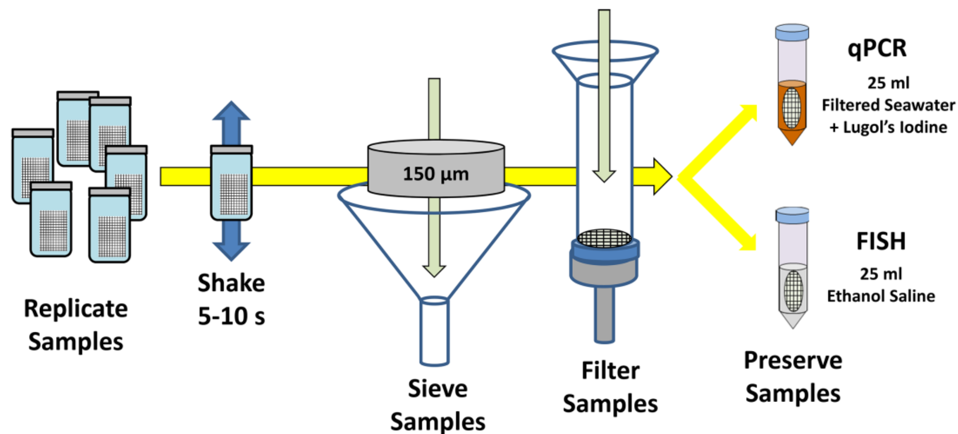

Fig. 2. Schematic of sample processing method for *Gambierdiscus* monitoring using the artificial substrate (AS) method in combination with species-specific qPCR and FISH quantification..

caribaeus, *G. carolinianus*, *G. carpenteri* and *Gambierdiscus* ribotype 1 (Pitz *et al*. 2014).

Dicussion

A major benefit of the qPCR method is high throughput. After DNA extraction and initial setup, assays for multiple *Gambierdiscus* species can be run with little additional effort. This is a great advantage for monitoring applications with multiple *Gambierdiscus* species and multiple sample sites with replicate samples. The major limitation of qPCR is that the method is semi-quantitative. Assays can readily discriminate cell concentrations when abundance is relatively high and perform very well for detecting species when abundance is low (1-2 cells sample^{-1}), but quantification is more variable when <10 cells are present sample^{-1} (Vandersea *et al*. 2012). This variation is due to slight differences in extraction efficiencies, changes in DNA over the cell cycle, and other factors (Bustin *et al*. 2009, Hariganeya *et al.* 2013). Despite this limitation, the high sensitivity of qPCR makes the method ideal for tracking *Gambierdiscus* blooms long before they develop.

The FISH method involves direct counts of *Gambierdiscus* cells using microscopy and may be more precise than qPCR for determining absolute cell concentrations when abundance is high. Similar to qPCR, FISH can accurately measure cell concentrations over a large dynamic range, but because it relies on cell counts, accuracy declines rapidly at low cell numbers due to increased sampling error (Rott *et al*. 2007, Litaker *et al.* 2010, Reguera *et al*. 2011). Other technical issues can arise including weak labeling of target cells, loss of cells during sample preparation, and masking of labeled cells by autofluorescence and particulate matter (Kim *et al.* 2005, Touzet *et al*. 2008, Chen *et al*. 2011). Some of these problems can be alleviated with careful optimization of the assays, decolorization of autofluorescent compounds, or by using alternate fixatives. Other potential improvements include peptide nucleic acid (PNA) probes, which offer stronger binding and greater hybridization efficiency, or by using internal controls of cultured cells (Chen *et al*. 2011, Toebe *et al*. 2012).

Each of these molecular methods may be utilized to quantify the abundance of *Gambierdiscus* species in monitoring samples, although they are relatively inaccurate when cell abundance is low. The most appropriate method depends on the resources of the monitoring program, and the number of *Gambierdiscus* species present. If only 1-2 species occur at the monitoring sites, then screening with a few species-specific qPCR or FISH assays may be sufficient to provide coastal managers with a reliable warning during the early stages of *Gambierdiscus* bloom development. If >2 species co-occur, the qPCR method is more practical for routine monitoring because of the method's high throughput, with the option to include FISH analyses when a bloom is developing. Once qPCR data indicate abundance of a toxic *Gambierdiscus* species exceeds a target threshold (e.g., 1000 cells 100 cm^{-2}), FISH assays for the targeted species may provide a precise measure of cell concentration necessary for regulatory action (i.e., fishing closures in affected areas, restriction on sales and distribution of fish). These species-specific techniques are best integrated into a formal *Gambierdiscus*

monitoring program using the artificial substrate method.

In summary, we propose that the AS method be employed in combination with species-specific molecular assays as a cost effective means of detecting and mitigating CFP risks associated with *Gambierdiscus* blooms in the Caribbean. The combined cell abundance and species quantification methods described above may be simplified further as more information becomes available about the toxicity of individual *Gambierdiscus* species. If ongoing research indicates that only a few *Gambierdiscus* species contribute to the flux of ciguatoxins into the marine food web, then a small number of molecular assays may be sufficient for advanced warning of CFP risks. This two-tiered monitoring approach can also be used for broad-scale screening of reefs for overal CFP potential and to follow geographic changes in *Gambierdicus* abunance patterns associated with climate change.

Acknowledgements

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HAB Technologies

Plankton Toolbox – open source software making it easier to work with plankton data

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Abstract

Modern database systems make it possible to access large data sets on the abundance and the biodiversity of phytoplankton including harmful algal bloom species. This gives insights into geographical distribution of species, bloom development etc. Large data sets may be time consuming to work with. We have developed Plankton Toolbox, an open source stand-alone application, to make the process easier. The software is mainly aimed at working with data from water samples analysed microscopically but it is applicable also to other data. The work flow includes importing data, a quality control step, ways to filter out data for specific purposes, functions for aggregating data, plotting tools and tools for statistical analyses. Importing and exporting of data in different formats is supported. For advanced statistical analyses exporting to other software is useful. The system is based on quality controlled species lists available at http://nordicmicroalgae.org but users are free to work with their own check lists. Lists on cell volume and carbon content of species come from the HELCOM-Phytoplankton Expert Group but users can also define their own lists. The software was developed using Python and runs on personal computers with Microsoft Windows or MacOS (Apple Inc.). The software is available at http://nordicmicroalgae.org/tools.

Keywords: phytoplankton, data analysis, data base, software, biovolume, statistics

Introduction

Investigating the abundance, biodiversity and biomass of phytoplankton are part of short term scientific investigations and long term monitoring programs in many areas world-wide. The long time series data is valuable e.g. for research related to climate change. The large data sets produced include data on the occurrence of harmful algal bloom species. This gives insights into geographical distribution of species, bloom development etc. For countries in the European Union (EU) legislation stipulates phytoplankton monitoring as part of the work aiming for good environmental/ecological status in lakes and in coastal seas. EU regulations concerning harvesting of bivalves include requirements for monitoring of biotoxin producing algae such as several dinoflagellates and some diatom species. Also conventions for protection of the marine environment such as OSPAR (Convention for the Protection of the Marine Environment of the North-East Atlantic – Oslo-Paris Commission) and HELCOM (Baltic Marine Environment Protection Commission - Helsinki Commission) include phytoplankton monitoring. The data on the abundance, biodiversity and biomass of phytoplankton is often stored in databases at national or international data centres. In recent years data is increasingly becoming freely available on the Internet making it accessible to the scientific community. In the Baltic Sea area, most of the data include the abundance and biodiverstiy of phytoplankton and also cell volumes at the species level, sometimes at the sub species level (forma, variety and subspecies). Working with these large datasets may be time consuming if common tools such as spread sheet software are used. Developing algorithms in open source programming languages such as R (http://www.r-project.org/) and Python (http://www.python.org) is also possible but often outside the field of knowledge for scientists specializing in phytoplankton research. We have developed Plankton Toolbox (Fig. 1), an open source stand-alone application, to make the process easier. Also data on zooplankton is within the scope of Plankton Toolbox. To the authors knowledge no similar tool is available at present. Inspiration comes partly from the field of physical oceanography where the open source software Ocean Data View (Schlitzer 2014) is used extensively.

A. Lincoln MacKenzie [Ed]. *Marine and Freshwater Harmful Algae 2014*. Proceedings of the 16th International Conference on Harmful. Algae. Cawthron Institute, Nelson, New Zealand and the International Society for the Study of Harmful Algae (ISSHA)

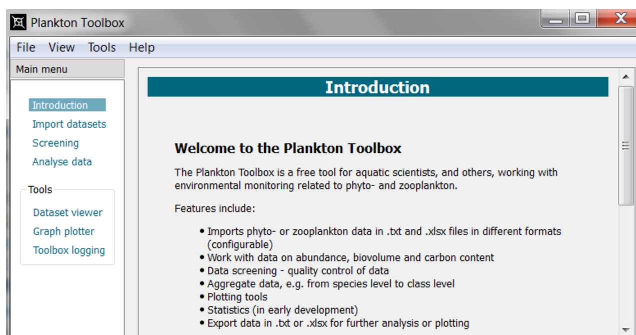

Fig. 1. Screen shot of the start page for the sofware Plankton Toolbox (Windows version).

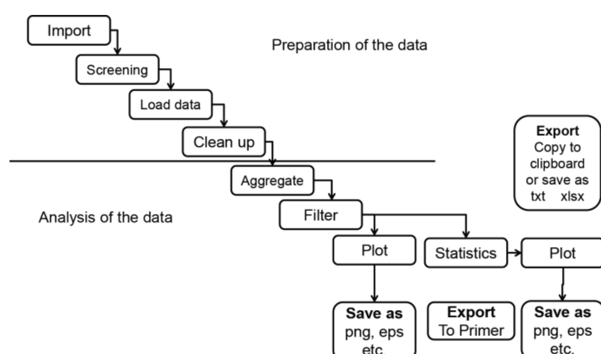

Fig. 2. An illustration of the step-wise processing of data in Plankton Toolbox.

Material and Methods

The software Plankton Toolbox (PTBX) was developed using open source software, i.e. Python version 2.7. The code, which is free as defined by the MIT-license, the Open Source Initiative, http://opensource.org/licenses/mit-license.php, is available at http://code.google.com/p/plankton-toolbox/. A version control system for keeping track of different versions of the code is used.
PTBX is available for download at http://nordicmicroalgae.org/tools. At present (1 March 2015) version 1.01 is current. PTBX can import data in many formats. Example datasets with phyto- and zooplankton data downloaded from the Swedish Oceanographic Data Centre (http://sharkweb.smhi.se) is part of the software package.
One of the features of Plankton Toolbox is the ability to aggregate data to different taxonomic levels, e.g. to class level. This requires a taxonomic tree, i.e. a hierarchy. The one supplied with the package is also used in the web site Nordic Microalgae, http://nordicmicroalgae.org and available for download there. AlgaeBase, http://algaebase.org (Guiry and Guiry 2015) is the main source of taxonomic information The tree is user selectable, i.e. the user can use a tree of his or her own choice. Another feature of PTBX is the ability to work with biovolumes of phytoplankton. A list of cell volumes for phytoplankton taxa from the Baltic Sea region based on Olenina *et al.* (2006) is supplied with PTBX. This list is updated yearly by the HELCOM Phytoplankton Expert Group and is available for download at www.ices.dk. The list also includes information on the trophic type of the organisms, e.g. autotrophic, mixotrophic or heterotrophic. The latter may be considered micro-zooplankton. In addition to the standardised lists support for lists handling synonyms and user defined lists is part of PTBX. Calculation of carbon content is part of PTBX. The equations used for phytoplankton were developed by Menden-Deuer and Lessard (2000).

Internal model

Internally PTBX operates only on tree oriented datasets. Nodes in the tree structure representing a dataset are:

- Dataset - one for each imported dataset.
- Visit - one for each sampling event (place/position and date/time).
- Sample - one for each sample or unit for analysis.
- Variable - a number of parameter values from the sample.

Each node contains a key/value list to handle field values.

Parsers

Parsers are used to translate from table oriented data to tree oriented data, and they are executed when importing datasets. Parsers are declared in Excel and can be modified by the user.

Results and Discussion

The Plankton Toolbox is a free tool for aquatic scientists, and others, working with phyto- and zooplankton data. It is available for MacOS and Windows. PTBX makes it possible to work with large data sets on the diversity, abundance, biovolume and carbon content of plankton efficiently. The software is useful for working with datasets emanating from quantitative and qualitative analyses of phytoplankton and zooplankton. Phytoplankton, including harmful

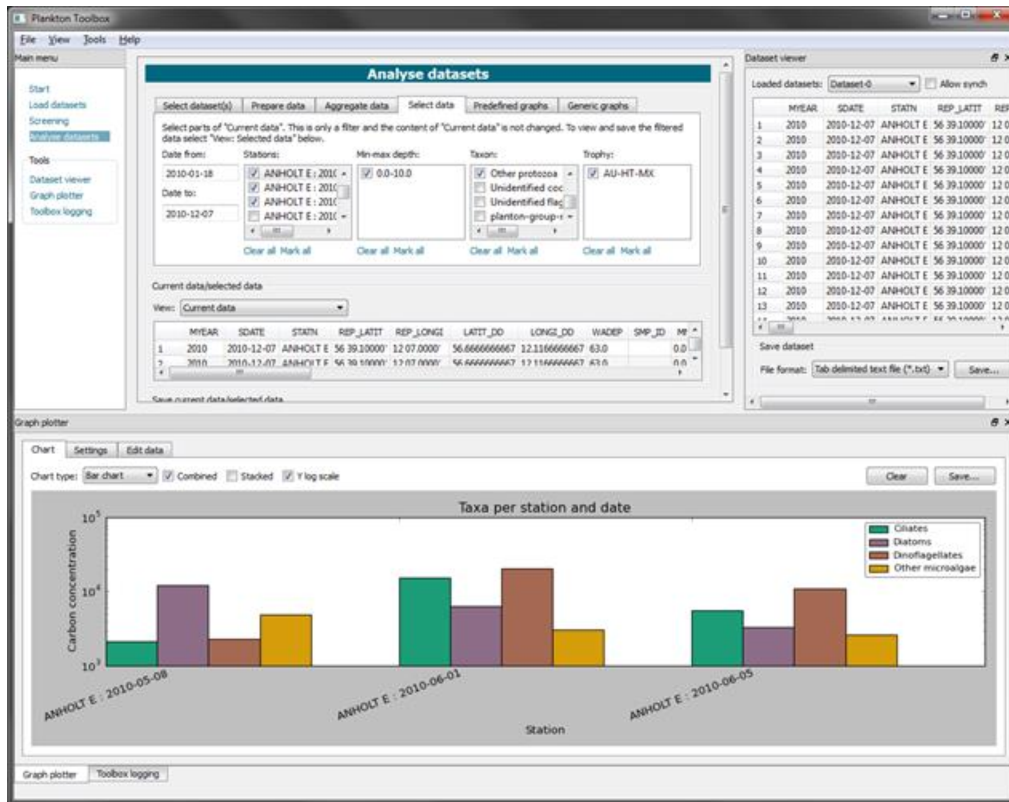

Fig. 3 Screen shot illustrating some functions in Plankton Toolbox

algae, are enumerated and identified in numerous ways; see e.g. Karlson *et al.* (2010). One of the most popular quantitative methods is water sampling, preservation of the sample and subsequent microscope analysis using the sedimentation chamber method (Utermöhl 1931, 1958; Edler and Elbrächter 2010). A more recent method is imaging flow cytometry (Chisholm and Olson 2007; Dubelaar *et al.* 2004; Poulton and Martin 2010). These methods produce data on the abundance (unit is often cells L^{-1}) of plankton taxa. The cell volume of the taxa is also often included to facilitate the calculation of biomass. PTBX offers a work flow for calculating biovolume of taxa (unit is often mm^3 L^{-1}) and also carbon content based on the algorithms by Menden-Deuer and Lessard (2000). Data from molecular methods may also be applicable. The step wise processing of data in PTBX is illustrated in Fig. 2 and described hereafter.

Importing data
PTBX imports data in text (txt) and Microsoft Excel (xlsx) files in different formats. Since data formatting differs depending on the source of the data importing formats are user configurable through parsers. A useful function is that data from different sources, in different formats, can be combined by importing multiple files. The data can then be exported as one consistent dataset in txt or xlsx format by the user.

Data screening - quality control of data
When the data has been imported the raw data can be screened in different ways to check for duplicate data, look for unrealistic dates, positions etc. There are also plotting tools to visualize the raw data.

Clean up
In this step unwanted data can be removed. One example is to remove data from a certain depth or data from localities that is not needed for the data analysis.

Aggregate
At this step data can be aggregated to different taxonomic levels, e.g. from species level to class level. Here also a function for adding zeros for organisms that are not observed in a sample is found. The software looks through all the sampling locations, dates and depths, creates a complete list of taxa observed in all the samples in the dataset, and adds zeros in abundance for a taxa that were not observed.

Filtering

In PTBX filtering means that the user may work interactively with the data; selecting one or a few taxa, selecting certain samples etc.

Plotting tools
Tools for plotting data in various ways are provided, e.g. for date vs. abundance or biovolume. The current aim of the plotting tools is to give the user the ability to produce fairly simple graphs. For publication quality output it is often necessary to export the data and use some graphical software package.

Statistics
Some statistical functions are part of PTBX. This part is planned to be developed further in future releases of the software.

Export
At most steps in the data processing data can be copied to the clipboard and pasted into other software running on the computer used. Data may also be exported in txt or xlsx formats for further analyses or plotting using other software.

Future
PTBX will be continuously updated according to requests from users. In the planning stage are support for working with the biomass of meso-zooplankton and more statistical features. A future update with functionality for using PlanktonToolbox as a counting tool by the microscope is being considered. Open source software is important for the plankton research community. PTBX is a new tool in the open source toolbox. Existing free tools include automated image analysis software (Gorsky *et al.* 2010; Schulze *et al.* 2013). Free access to data and the tools for working efficiently with the data will make it possible to address scientific problems previously difficult to work with.

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A novel yeast bioassay for the detection of marine microalgae biotoxins based on tunicate xenobiotic receptors

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Abstract

Xenobiotic receptors regulate transcription of genes involved in metazoan detoxification pathways. Tunicate (phylum Chordata) xenobiotic receptors may have adaptively evolved to bind marine bioactive compounds, including microalgal biotoxins. To investigate this idea, we utilised tunicate xenobiotic receptors as sensor elements in bioassays. Recombinant yeast strains were developed that express chimeric proteins combining tunicate xenobiotic receptor ligand-binding domains (LBDs) along with a generic DNA-binding domain. These proteins mediate ligand-dependent transcription of a reporter gene (*lacZ*) encoding β-galactosidase, an enzyme which can be easily assayed. Bioassay strains corresponding to xenobiotic receptor LBDs from two tunicates (*Ciona intestinalis* and *Botryllus schlosseri*) were exposed to five natural aquatic toxins. Three marine microalgal biotoxins (okadaic acid, pectenotoxin-11 and portimine) activated the bioassays at nanomolar concentrations (EC_{50} = 16 – 883 nM). These EC_{50} values were up to three orders of magnitude lower than synthetic tunicate xenobiotic receptor LBD agonists. These findings are consistent with the hypothesis that tunicate xenobiotic receptors have adaptively evolved to bind commonly encountered natural marine biotoxins. Given the large number of tunicate species, occupying a wide range of ecological niches, we propose that tunicate xenobiotic receptor bioassays may be suitable 'sensor elements' for the detection of marine microalgal biotoxins.

Keywords: Ciona intestinalis, *Botryllus schlosseri*, pregnane X receptor, *Saccharomyces cerevisiae*, marine biotoxins, bioassay

Introduction

Marine microalgae can produce highly toxic chemicals that cause widespread poisoning within marine ecosystems (Wang 2008; MacKenzie *et al.* 2011). Internationally accepted methodologies for the detection of such natural marine biotoxins are increasingly based on chemical, rather than biological, assays (Suzuki & Quilliam 2011; McNabb *et al.* 2012). Although chemistry-based detection methods are reliable and highly specific, they require detailed knowledge of the targeted biotoxin structures, making detection of unknown marine biotoxins challenging (Humpage *et al.* 2010; Nicolas *et al.* 2014). Consequently, there is a need for simple, robust and inexpensive bioassays for microalgal biotoxin detection (Vilarino *et al.* 2010; Nicolas *et al.* 2014).

The vertebrate pregnane X receptor (PXR) is a ligand-dependent nuclear receptor which regulates transcription of multiple genes involved in detoxification pathways (Kliewer *et al.* 2002; Wallace & Redinbo 2013; Xie & Chiang 2013). Known vertebrate PXR ligands are structurally diverse and include dietary xenobiotics (e.g. synthetic and herbal drugs, environmental chemicals; Chang & Waxman 2006; Staudinger *et al*. 2006; Hernandez *et al.* 2009). Comparisons of the ligand-binding domain (LBD) sequences of vertebrate PXR orthologues suggest that they may have adaptively evolved to bind xenobiotics that are typically encountered by a given taxon (Krasowski *et al*. 2005a, b).

Filter-feeding marine tunicates (phylum Chordata, sub-phylum Urochordata) are exposed to a wide range of microalgal biotoxins associated with their diet (Sekiguchi *et al.* 2001; Roje-Busatto & Ujević 2014). Tunicates also form the sister clade to the Vertebrata (Delsuc *et al.* 2008). Annotated genomes of two ascidian tunicates, *Ciona intestinalis* and *Botryllus schlosseri*, encode at least two genes orthologous to the vertebrate PXR and vitamin D receptor (VDR), abbreviated as VDR/PXR (Dehal *et al.* 2002; Yagi *et al.* 2003; Voskoboynik *et al.* 2013). It has been shown that

Table 1. Activation of bioassays by five algal toxins. Ligand-dependent induction of β-galactosidase enzymatic activity was measured in yeast strains carrying plasmids pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 or pGAL4.*Bs*LBD.VP16. Mean effective concentrations (EC_{50} values) and 95% confidence intervals (95% CI) are given in nM. 95% CIs represent variation within a triplicate measurement.

Comp. Name	Supplier (Catalogue No.)	pGAL4.*Ci*LBDΔ31.VP16		pGAL4.*Ci*LBD.VP16		pGAL4.*Bs*LBD.VP16	
		EC_{50}	95% CI	EC_{50}	95% CI	EC_{50}	95% CI
Okadaic acid	Sapphire Bioscience (AB120375)	19	1.1 – 327	27	15.4 – 46.4	16	8.3 – 30.4
Pectenotoxin-11	MacKenzie 2013	633	117 – 3437	883	134 – 5834	553	3.4 – 90,840
Portimine	Selwood *et al.* 2013	143	114 – 180	130	105 – 162	124	77 – 202
Microcystin-RR	DHI Lab Products (PPS-MCRR)	DNC	DNC	DNC	DNC	DNC	DNC
Anatoxin-A	NRC Canada (IMB-CRM-ATX)	DNC	DNC	DNC	DNC	DNC	DNC

Abbreviations: DNC, did not compute.

one VDR/PXR gene from *C. intestinalis* (*Ci*VDR/PXRα, GenBank acc. no. NM_001078379) can be activated by microalgal biotoxins (okadaic acid and pectenotoxin-2) when expressed in mammalian cell lines (Reschly *et al.* 2007; Ekins *et al.* 2008; Fidler *et al.* 2012).
Here we report the development of recombinant yeast (*Saccharomyces cerevisiae*) strains expressing the *C. intestinalis* and *B. schlosseri* VDR/PXRα LBD as fusion proteins combined with the GAL4 DNA-binding domain (GAL4-DBD) and a generic transcription activation domain (VP16-AD). These chimeric proteins mediate ligand-dependent transcription of a reporter gene (*lacZ*). The bioassay strains were tested with three microalgal biotoxins and two cyanobacterial toxins. Results indicated that tunicate VDR/PXRα LBD-based recombinant yeast bioassays are technically feasible. These bioassays may find application in high-throughput screens for microalgal biotoxins.

Materials and Methods

Compounds tested in the bioassay are listed in Table 1. All chemicals were dissolved in analytical grade ethanol (Merck, Whitehouse Station, U.S.A.) to form stock solutions. Serial dilutions were added to the bioassay media at a final ethanol concentration of 1% (v/v).
Detailed protocols for media preparation, generation of plasmid constructs and bioassay procedures are described in Richter & Fidler (2015). All the plasmid constructs used in this work were derived from the 7.3 kb yeast expression vector pGBKT7 (Clontech Laboratories Inc., Mountain View, U.S.A., Fig. 1). The recombinant yeast (*S. cerevisiae*) strains express chimeric genes that encode proteins combining one or more of three basic elements: (i) a GAL4-DBD (147 residues); (ii) a predicted tunicate VDR/PXRα orthologue LBD (*C. intestinalis* VDR/PXRα: 312 residues; *C. intestinalis* VDR/PXRα C-terminal truncated LBD: 281 residues; *B. schlosseri* VDR/PXRα: 305 residues) and; (iii) a VP16-AD (68 residues, Fig. 1) derived from herpes simplex virus protein 16 (Louvion *et al.* 1993).

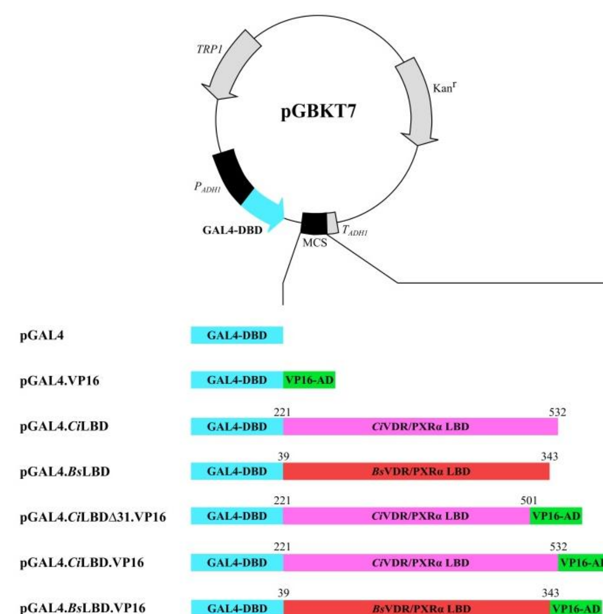

Fig. 1. Schematic representation of the plasmid encoded fusion genes/chimeric proteins generated in this study (Richter & Fidler 2015). *Abbreviations*: MCS, multiple cloning site; *Bs*VDR/PXRα, *B. schlosseri* VDR/PXRα (red); *Ci*VDR/PXRα, *C. intestinalis* VDR/PXRα (pink); GAL4-DBD, GAL4 DNA-binding domain (blue); LBD, predicted ligand-binding domain; VP16-AD, viral protein 16 activation domain (green).
The yeast host strain (PJ69-4A; James *et al.* 1996) encodes a *lacZ* reporter gene under the control of a GAL4-regulated promoter. Binding of a

compound to the tunicate VDR/PXRα LBD results in binding of the chimeric protein to the GAL4-regulated promoter, activating transcription of the adjacent *lacZ* gene. Thus, the recombinant yeast strains that produce increased amounts of the enzyme β-galactosidase (encoded by *lacZ*) in response to potential tunicate VDR/PXR agonists can be used as bioassays (Richter & Fidler 2015).

Results and Discussion

Pursuing the hypothesis that the ecologically relevant ligands of tunicate VDR/PXRα receptors include those toxins which are naturally present in a marine filter-feeder's diet, three established microalgal biotoxins (okadaic acid, pectenotoxin-11 (PTX-11) and portimine) were tested in the bioassays.

All three microalgal biotoxins induced β-galactosidase enzymatic activity from those strains carrying plasmids encoding a tunicate VDR/PXRα LBD fused to the VP16-AD: pGAL4.*Ci*LBDΔ31.VP16, pGAL4.*Ci*LBD.VP16 or pGAL4.*Bs*LBD.VP16 (Figs. 1 and 2, Table 1). In contrast, yeast strains carrying the two negative control plasmids (pGAL4 or pGAL4.VP16) showed no evidence of induced β-galactosidase enzymatic activity nor did the two strains encoding GAL4.VDR/PXRα LBD fusion proteins that lacked a C-terminal VP16-AD domain (Fig. 2). There was no indication of toxicity of microalgal biotoxins towards the yeast cells within the concentration ranges tested (data not shown). Two freshwater cyanobacterial toxins (microcystin-RR and anatoxin-A) were also tested in the bioassays. These compounds were considered 'negative controls' as, being of freshwater origin, it is unlikely that they would have been encountered by marine tunicates over evolutionary time. As expected, both compounds were inactive in the bioassays (Table 1).

Identification of portimine from the pinnatoxin-producing dinoflagellate Vulcanodinium as an activator of both tunicate VDR/PXRα LBDs tested was consistent with its reported toxicity when tested using mammalian cells *in vitro* (LC_{50} = 2.7 nM), suggesting that portimine may be toxic towards a wide range of chordates— including tunicates (Selwood *et al.* 2013).

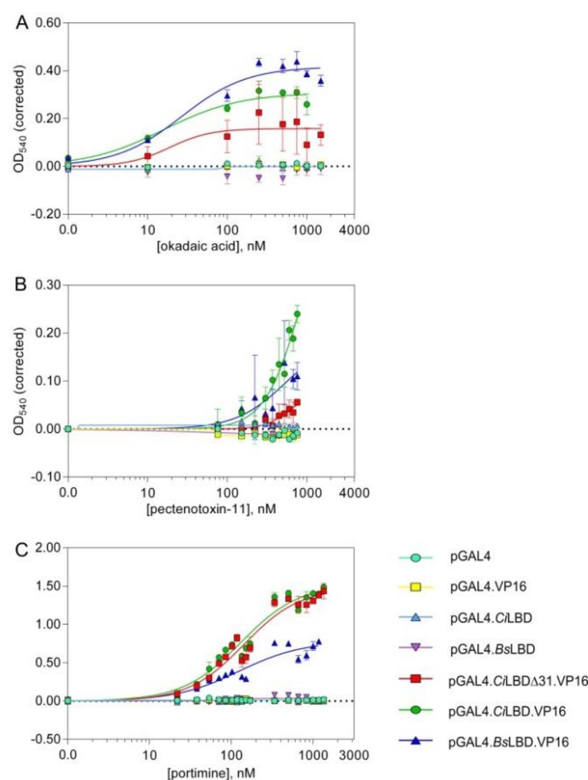

Fig. 2. Activities of okadaic acid (**A**), pectenotoxin-11 (**B**) and portimine (**C**) tested in the yeast bioassays. β-galactosidase enzymatic activities (measured by $OD_{540\ (corrected)}$ values) were determined after 48 hours. Data points represent means of three replicates. Error bars show ± one standard deviation. Dotted lines indicate baseline.

The yeast strain expressing the shorter version of the *C. intestinalis* VDR/PXRα LBD (pGAL4.*Ci*LBDΔ31.VP16, Fig. 1) was more strongly activated by portimine than the strain expressing the longer version (pGAL4.*Ci*LBD.VP16, Fig. 2C). However, this difference was not reflected in corresponding EC_{50} values (Table 1). In contrast, the yeast strain expressing the shorter version of the *C. intestinalis* VDR/PXRα LBD was less strongly activated by okadaic acid and PTX-11 (Fig. 2A–B) but again this difference was not reflected in EC_{50} values (Table 1). The EC_{50} values for activation of the full-length *C. intestinalis* VDR/PXRα LBD by okadaic acid (pGAL4.*Ci*LBD.VP16 EC_{50} = 27 nM, Table 1) is comparable with a value reported using mammalian cell line *Ci*VDR/PXRα-based bioassays (EC_{50} = 18.2 nM) supporting the validity of the yeast bioassay results (Fidler *et al.* 2012).

Two of the microalgal biotoxins that activated the tunicate VDR/PXRα LBDs in this study (okadaic

acid and PTX-11) are produced by dinoflagellate species within the cosmopolitan genus *Dinophysis*, which can reach cell densities of 10^2 – 10^5 cells/L in coastal marine waters (Reguera *et al.* 2012). Thus, it is to be expected that filter-feeding marine invertebrates will encounter and accumulate such biotoxins through their diet (Sekiguchi *et al.* 2001; Roje-Busatto & Ujević 2014). Although the toxicity of microalgal biotoxins towards tunicates requires more investigation, the yeast bioassay data presented here is consistent with the speculation that tunicate VDR/PXRα LBDs have adaptively evolved to bind commonly encountered marine biotoxins (Fidler *et al.* 2012). Given the highly modular structure of the chimeric proteins expressed, along with the vast taxonomic and ecological diversity of filter-feeding marine organisms (Nishida *et al.* 2010), it is possible to envisage a virtually unlimited number of analogous bioassays utilising orthologous receptor LBDs from marine organisms. By selecting xenobiotic receptor LBDs on the basis of an organism's phylogeny and ecology, bioassays with differing ligand specificities could be developed for the detection of a wide range of microalgal biotoxins (Richter & Fidler 2014).

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Improvements to the RTgill-W1 fish gill assay for ichthyotoxins: A comparison of the potency of different toxin fractions and extracts tested with different microplate materials

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Abstract

Different microplate materials were compared for *in vitro* screening of purified phycotoxins and algal extracts using the fish gill cell line RTgill-W1. Glass plates coated with collagen I were more suitable for assays with brevetoxin and for methanol extracts of the haptophyte *Prymnesium parvum* (up to 33% more toxic). By contrast, polystyrene plates showed better results with karlotoxin (19% more toxic), and with extracts from the dinoflagellates *Karlodinium veneficum*, *Karenia mikimotoi* and *Amphidinium carterae* (20-37% more toxic). Care should also be taken to conduct tests in light or dark conditions when toxins or extracts are light sensitive, as was found with *Chattonella, Fibrocapsa* and *Prymnesium* extracts (53-90% difference among results). As a first screening step for unknown toxins the use of both glass and plastic plates is strongly recommended. The differential behaviour of ichthyotoxins in plastic or glass containers and in light and dark conditions also contributes to our understanding on how these compounds can mediate fish kills in nature.

Keywords: Harmful phytoplankton; toxins; algal extracts; gill cells; RTgill-W1; ichthyotoxicity.

Introduction

The potency of intra- and extracellular phycotoxins and their extracts can be highly variable depending on analytical test conditions. Plastic materials are common in laboratories since they are cheap and disposable, even though adsorption of some toxins is of concern. Microcystins from cyanobacteria possess hydrophilic groups as well as a hydrophobic residue (Rivasseau et al., 1998). Adsorption of such toxins by laboratory materials has been claimed to occur due to hydrophobic interactions with plastic surfaces (Hyenstrand et al., 2001), although hydrogen bonding and physical surface properties such as porosity, roughness, and thickness may also contribute to toxin adsorption (Lee and Walker, 2008). A microplate-based assay using the fish cell line RTgill-W1 from rainbow trout was recently developed to test the effect of widely different algal extracts and ichthyotoxins (Dorantes-Aranda et al., 2011). Subsequent applications with this assay (He et al., 2012; Place et al., 2012; Zou et al., 2013; Sorichetti et al., 2014) have raised the question whether there is any influence of the microplate material on the toxic effect of algal toxins and extracts.

Material and Methods

The cell line RTgill-W1, initiated from gill filaments of rainbow trout *Oncorhynchus mykiss* (Bols et al., 1994), was obtained from the ATCC. Gill cells were grown in 25-cm^2 culture treated flasks with Leibovitz L15 medium, supplemented with fetal bovine serum 10% (v/v) and an antibiotic/antimycotic solution (Sigma). Cells were kept at 20°C in the dark. Gill cells were detached using a trypsin-EDTA solution (Sigma) for routine subculturing and plating. Brevetoxin (PbTx-2) was obtained from MARBIONC, and karlotoxin (KmTx-2) was kindly provided by Allen R. Place. Both toxins were resuspended in >99.7% methanol. Microalgae were obtained from the Harmful Algal Culture Collection of the Institute for Marine and Antarctic Studies, University of Tasmania (Table I). Algae were grown in GSe medium (Blackburn et al., 1989) at a salinity of 35 at 20ºC. Light was provided at 150

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μmol photons m^{-2} s^{-1} (cool white fluorescent lamps) with a light/dark cycle of 12/12 hrs. Aliquots of 30 mL of algal cultures in the late exponential growth phase were centrifuged (1000 rpm, 5 min), and the pellet resuspended in 2 mL of analytical grade MeOH (>99.7%). Samples were sonicated for 10 min on ice and centrifuged (3000 rpm, 10 min). The supernatants were recovered and stored at -80ºC.

Table I. Microalgae used for the preparation of extracts at their respective concentration.

Species	Cell concentration ($\times10^3$ cells mL^{-1})
Raphidophytes	
Chattonella marina	47.5
Fibrocapsa japonica	57.5
Heterosigma akashiwo	240
Dinoflagellates	
Amphidinium carterae	67.5
Karenia mikimotoi	570
Karlodinium veneficum	235
Haptophyte	
Prymnesium parvum	1377
Chlorophyta	
Tetraselmis suecica	200

Three types of 96-well microplates were used: glass plates (Zinsser Analytic) made of borosilicate glass, clear transparent plastic plates (Greiner bio-one) and black plastic plates with transparent bottom (Nunc) both made of polystyrene. The plastic plates were obtained sterile and pre-treated for cell culturing. Glass plates were washed with sterile MQ water, autoclaved and coated with collagen I from rat tail (Sigma) to improve cell attachment. Collagen was dissolved in acetic acid (0.1 M) and used at concentrations of 0, 125, 250, 500, 750 and 1000 μg mL^{-1} to determine the concentration for optimal cell attachment. Collagen was added to plate wells in quadruplicate, and incubated for either 1 or 24 hrs. Collagen was discarded and plates rinsed twice with sterile PBS. Plates were left to dry under sterile conditions and UV radiated for 15 min. Confluent gill cells were detached and seeded in microplates in quadruplicate wells at 2×10^5 cells mL^{-1}. Cells were allowed to attach for 48 hrs prior to the experiments. Experimental concentrations of toxins and extracts were: purified brevetoxin 0.1-40 μg mL^{-1}; karlotoxin 1-10000 ng mL^{-1}; extracts 0.2-2.0 %. Toxins and extracts were diluted with L-15/ex (Schirmer et al., 1997) to achieve these concentrations. MeOH final concentration was 2 and 10% for the algal extracts and toxins, respectively. Cells were rinsed with PBS and exposed to toxins and algal extracts for 2 hrs. As a vehicular control, MeOH was diluted with L-15/ex to yield 2 and 10% (v/v). After the 2 hrs exposure, experimental solutions were discarded and gill cells were rinsed with PBS. Resazurin (Sigma) was used to determine cell viability. We had used alamar blue (resazurin-based indicator dye) in our previous work to determine cell viability (Dorantes-Aranda et al., 2011). Resazurin and alamar blue showed comparable results in our preliminary studies. Resazurin was prepared as per Penney et al. (2002). Gill cells were incubated in L-15/ex with resazurin 5% (v/v) for 2 hrs in the dark. Fluorescence signal was detected at 540 and 590 nm of excitation and emission, respectively, in a microplate reader (BMG Labtech). Viability is expressed as percentage of the readings compared to the controls, which represent 100% viability.

Analysis of variance (ANOVA) was used to determine any differences among experimental groups. Normality of the data was determined with Kolmogorov-Smirnov test, and homoscedasticity with Levene's test. *Post hoc* Tukey tests were performed when multiple comparisons were required. A significance level of 95% (α=0.05) was employed. Statistical analyses were conducted in Statistica 12 (StatSoft Inc.).

Results and Discussion

Collagen I worked effectively as a coating agent to pre-condition plates for gill cell attachment. Five concentrations of collagen at two incubation times, 1 and 24 hrs, did not show significant differences in cell attachment, even when compared with the control cells growing in conventional plastic culture-treated plates. Cells growing in wells without collagen did not show any differences compared to the cells in coated wells. Thus a further experiment of cell growth over a 10 day period was performed coating the glass plates with collagen for 1 hr (seeding gill cell concentration was 5×10^4 cells mL^{-1}). Although lower fluorescence was detected in uncoated wells, there were no significant differences with those growing in wells with 125-500 μg collagen mL^{-1}. However, they were significantly different to the cells from the control plastic plate. An incubation period of 1 hr with a collagen concentration of 250 μg mL^{-1} was chosen

for coating the glass plates. Brevetoxins, karlotoxin and algal extracts showed variable toxicity towards the gill cells. Gill cell viability generally decreased with increasing extract or toxin concentration. Critically, the toxic response was variable among the three types of microplates used. Brevetoxin showed a higher impact on gill cell viability when the exposure was performed in glass plates, especially at concentrations of ≥10 μg mL^{-1} (Fig. 1).

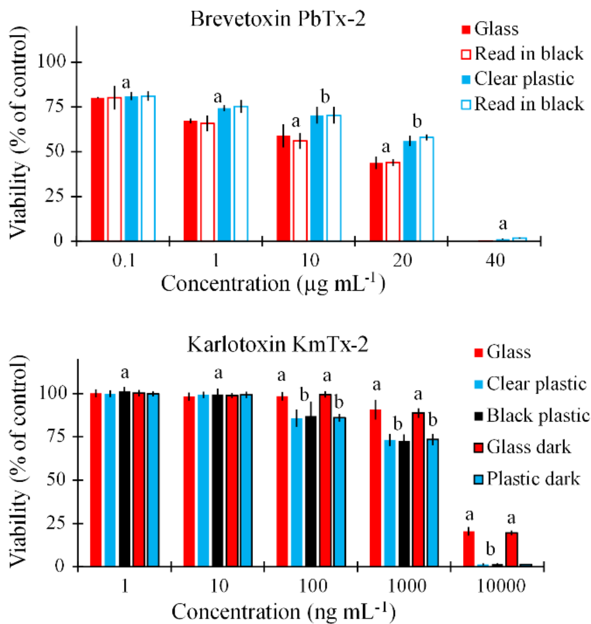

Fig. 1. Comparative effect of brevetoxin and karlotoxin on gill cell viability using glass and plastic plates. Karlotoxin was further tested in black plastic plates in light and dark conditions. Letters on columns indicate significant differences ($p<0.05$) among plate types at each concentration (i.e. treatments with "a" are different from "b"). Error bars represent the standard deviation of quadruplicate wells.

No viable gill cells remained in either clear plastic or glass plates after exposure to 40 μg mL^{-1} brevetoxin. However, at concentrations of 10-20 μg mL^{-1}, gill cell viability was significantly lower in exposures conducted in glass rather than plastic plates (11-12% lower), suggesting binding of brevetoxin to the plastic material. Conversely, karlotoxin toxicity towards the gill cells was higher in plastic than in glass plates (≥100 ng mL^{-1}). While a complete loss of cell viability was observed at a karlotoxin concentration of 10000 ng mL^{-1} in the plastic plates, 20% of gill cells still remained viable in exposures conducted in glass plates. Thus glass materials appear to bind or adsorb karlotoxin. No difference in karlotoxin toxicity towards gill cells was detected between light and dark conditions, nor between clear-transparent and black plastic plates (Fig. 1). When resazurin containing medium was transferred from clear-walled glass and plastic to black-walled plates after incubation to test for fluorescence cross-contamination among adjacent wells, no significant differences were observed. These results are shown in Figure 1 for brevetoxin but the same pattern was observed in all experimental conditions.

Methanol extracts from the raphidophytes *Chattonella marina* and *Heterosigma akashiwo* showed comparable toxic effects when using glass and clear plastic plates in light conditions (Fig. 2). Extracts tested in black plastic plates or glass and clear plastic plates kept in the dark did not negatively influence cell viability. *Fibrocapsa japonica* extracts showed a similar pattern as the two previous raphidophytes, except that extracts (≥0.7%) in glass plates in dark conditions showed a slightly higher toxic effect compared to black and clear plastic plates (darkness). Extracts from the haptophyte *Prymnesium parvum* exhibited a higher effect on gill cell viability in glass plates in both light and dark conditions. Cell viability was 22% lower in glass when compared to clear plastic plates at an extract concentration of 2% in light (Fig. 2). Negative control extracts from *Tetraselmis suecica* did not show any decrease in gill cell viability regardless of the type of microplate used. Methanol extracts of *Karenia mikimotoi*, *Karlodinium veneficum* and *Amphidinium carterae* proved more toxic in plastic plates, especially in clear plastic ones. No toxic effect by *K. mikimotoi* and *K. veneficum* extracts was observed when exposure was performed in black plates, or in glass and clear plastic plates in the dark, suggesting an important role of light in algal extract transformation and toxicity. *A. carterae* extracts proved to be more toxic in light than in dark conditions for both plate types. A maximum difference of 27, 34 and 20% gill cell viability was observed between exposures conducted in glass and clear plastic plates for *K. mikimotoi*, *K. veneficum*, and *A. carterae*, respectively.

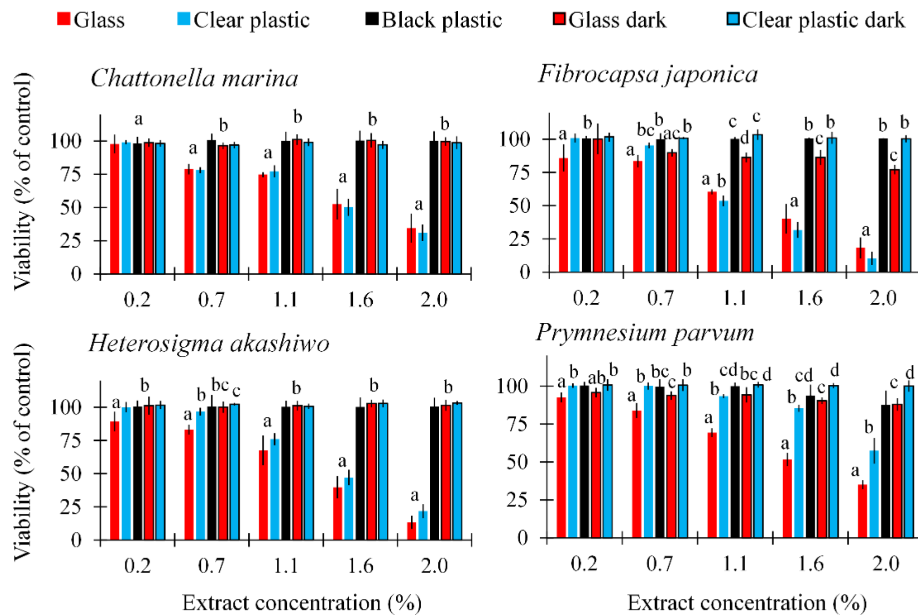

Fig. 2. Effect of methanol algal extracts on gill cell viability as influenced by microplate material under light and dark conditions.

Our results show that microplate materials can play an important role in assessing the toxic potency of ichthyotoxins and extracts when used in *in vitro* experimentation. The possible interference may be due to the chemical nature and properties of the compounds, such as hydrophobicity. For hydrophilic compounds, polystyrene plastic plates are recommended due to their hydrophobic surfaces not being able to bind or adsorb these compounds. For hydrophobic compounds (e.g. brevetoxin) glass plates are more suitable. However, as a first screening step for unknown toxins or extracts, use of both glass and plastic plates is strongly recommended. Finally, the differential behaviour of ichthyotoxins in plastic or glass containers in light or dark conditions also sheds light on how these compounds can mediate fish kills in nature. Blooms may be more toxic in sunny days, when there is more light, compared to cloudy or dark conditions.

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Application of solid phase adsorption toxin tracking (SPATT) in the East China Sea

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Abstract

The Zhejiang Coast along Eastern China suffers from recurrent and intense diarrhetic shellfish poisoning (DSP) outbreaks caused by *Dinophysis* species. A newly developed *in situ* method, solid phase adsorption toxin tracking (SPATT), is reported to be an effective tool to monitor the DSP and other lipophilic toxins. Here, we conducted a time series study to investigate the application of SPATT in East China Sea. During 11th of April to 4th of July 2014, the SPATT discs containing DIAION®HP-20 resin were suspended along the Coast of Gouqi Island, East China Sea on a 10-day harvest basis in conjunction with sampling of local mussels. Okadaic acid (OA), dinophysistoxin-1 (DTX1), homo-yessotoxin (homoYTX), pectenotoxin-2 (PTX2) and gymnodimine (GYM) were detected in both resins and mussels using liquid chromatography-tandem mass spectrometry (LC-MS/MS). No dinophysistoxin-2 (DTX2), yessotoxin (YTX), spirolide-1 (SPX1), brevetoxin-2 (PbTX2), PbTX3, azaspiracid-1 (AZA1), AZA2, AZA3 and domoic acid (DA) were found. Within the detectable toxins, OA, DTX1 and homoYTX were quantified. The OA+DTX1 toxin contents were lower than the international threshold (45 ng OA e.q./g shellfish meat), with the contents of 9.81 to 31.21 ng/g in the SPATT, and 2.71 to 20.45 ng/g in mussels, respectively. The OA+DTX1 content in the resin samples were obviously greater than that in mussels, indicating the SPATT (HP20) could be used as a sensitive monitoring tool for OA and DTXs in the local region. In addition, OA+DTX1 and homoYTX peaked in spring (April and May), suggesting that spring is the highest risk season for the DSP outbreaks in the Gouqi Island. Further long term investigations are required. All these results supported that SPATT has the potential to be used as an early warning tool to monitor the occurrence of the *Dinophysis* blooms and shellfish contamination events in this region.

Keywords: solid phase adsorption toxin tracking (SPATT), HP20, Diarrhetic shellfish poisoning (DSP), Okadaic acid (OA), Dinophysistoxin-1 (DTX1), homo-yessotoxin (homoYTX), LC-MS/MS

Introduction

The Zhejiang Coast of Eastern China is one of the world's main producers of mussels (*Mytilus* spp.) and other bivalves of commercial value. However, this region suffers from recurrent diarrhetic shellfish poisoning (DSP) outbreaks caused by *Dinophysis* species (Li *et al*. 2012), and the DSP outbreaks are becoming increasingly frequent and intense. Therefore, understanding the distribution and dynamics of DSP in this region has become an urgent scientific and managerial challenge that requires a multidisciplinary and site-specific approach. Traditional marine biotoxin monitoring programs, including the routine sampling and testing of shellfish and phytoplankton, are subjected to a series of serious limitations and disadvantages (MacKenzie *et al*. 2004, 2010; Fux *et al*. 2009; Rundberget *et al*. 2009; Lane *et al.* 2010). Therefore, developing a simple and sensitive tool for early warning of harmful algal blooms and shellfish contaminations is important and urgent to protect consumers and facilitate the management of local government.

The development of SPATT was first conducted by MacKenzie *et al.* (2004). Thereafter, the SPATT sampling technique was deployed in New Zealand, Australia, Norway, Spain, Ireland, and USA for a suite of lipophilic and hydrophilic phycotoxins including OA, DTXs, PTXs, YTXs, AZAs, DA, PSP, etc., using absorbents of Diaion®HP20 (MacKenzie *et al*. 2004, 2010; Fux *et al*. 2008; Turrell *et al*. 2007; Lane *et al*. 2010). In our study , HP20 was selected as the absorbent for the local lipophilic phycotoxins in East China Sea. The objectives of this study were to conduct

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a temporal and spatial analysis of the DSP associated toxins using SPATT in the Coast of Gouqi Island, East China Sea, to analyse the relationship of toxins in mussels and SPATTs, and to evaluate whether SPATT (HP20) could work as an effective universal early warning tool for micro-algae toxins in this region.

Methods

SPATT discs design and construction

The SPATT discs (Embroidery disc: 9 cm in diameter) were constructed by 120 μm SEFAR NITEX mesh (~23×12cm) and filled with HP20 resin (9.00±0.05 g dry weight). The resins were previously activated by immersing in 100% methanol (3 L) for 48 h. Then, they were rinsed in de-ionized water (MilliQ) and sonicated for 10 min to remove methanol residues through 10 min sonicating contained in 500 mL water. The discs were stored in MilliQ at 4-6 ℃ until deployment.

Field deployment and sample extraction

Three sampling sites, G1, G6 and G7 (30° 43.050' N, 122° 46.283' E), were set in the Coast of Gouqi Island, near the estuary of Yangtze River and Hangzhou Bay in East China Sea. The SPATT discs were suspended at ~2 m depth and retrieved every 10 days from 11 April 2014 to 4 July 2014. Meanwhile, mussels were collected in the same sites as SPATT discs were deployed. The disc and mussel samples were stored at -80 ℃ prior to extraction.

SPATT extraction method

SPATT discs were rinsed in 500 mL MilliQ water twice for 5 min, and shaken vigorously to remove salts and excess water. Then the resins were washed into empty 30 mL glass syringe which was previously located on a solid phase extraction (SPE) vacuum manifolds. Around 25 mL 100% methanol were added into the syringe to elute the resins at ca 1 mL/min flowing rate. The extracts were collected in 25 mL volumetric flasks. Extracts were heated at 40 °C in a heating block, dried under a stream of N_2, and re-suspended in 5 mL of methanol for toxin analysis.

Mussel extraction and hydrolysis

A portion of around 100 g mussel flesh (triplicate) was homogenized by a kitchen meat grinder. 2 g out of that 100 g homogenized flesh (wet weight) was mixed with 9 mL 100% methanol by vortex mixer for 1 min. Samples were centrifuged at 4000×g for 5 min. Repeat the extration of the residual tissue pellet with another 9 mL of 100% methanol, mixed and centrifuged. Two sets of supernatants were combined, and collected in 30 mL glass syringe. Drying and concentrating method were the same as that for SPATT.

Samples were hydrolyzed to detect and quantify the total content of OA and DTXs. The hydrolysis procedure was: incubating 1 mL of the extracts with 0.125 mL of 2.5 M NaOH at 76 ℃ for 40 min. The solutions were then cooled to room temperature, neutralised with the addition of 0.125 mL of 2.5 M HCl, filtered for toxin analysis.

LC–MS/MS analysis

The Dionex UltiMate 3000 LC system and AB 4000 mass spectrometer system with electrospray ionization was used for the analysis. Chromatographic separation was performed using a Waters XBridge^{TM}C18 column (3.0×150 mm, 3.5 μm particle size) at 40 ℃ for negative mode and Waters XBridge^{TM}C18 column (2.1×50 mm, 2.5 μm particle size) at 25 ℃ for positive mode. The mobile phase of negative mode consisted of phase A, 0.05 v/v % ammonia in water and phase B, 0.05 v/v % ammonia in 90% acetonitrile, with a flow rate of 0.4 mL/min and 10 μL injection. A linear gradient elution from 10% to 90% B was run for 9 min, held for 3 min at 90% B, decreased to 10% B in 2 min and held at 10% B for 4 min to equilibrate at initial conditions before the next run was started. For positive mode (flow rate: 0.3 mL/min), a linear gradient from 10% to 80% acetonitrile containing a constant concentration of buffer (2 mM ammonium formate and 50 mM formic acid) was run between 0 and 9 min, and held at 80% acetonitrile for 2 min. OA, DTX1,2, YTX and homeYTX were analysed in negative mode, and SPX1, PbTX2,3, PTX2, AZA1,2,3, GYM and DA were analysed in positive mode. Standard for all these toxins were purchased from the National Research Council, Canada.

Results and Discussion

The HP20 SPATTs were deployed in three sites along the Coast of Gouqi Island. Of all the examined toxins, OA, DTX1, PTX2, homeYTX and GYM were detected in both resin and mussel samples during 11 April to 4 July 2014. No DTX2, YTX, SPX1, PbTX2,3, AZA1,2,3 and DA were found. The contents of OA and DTX1 in SPATT were 1.26 to 18.10 ng/g and 0.85 to 15.04 ng/g, respectively. The same profiles but lower amounts of OA and DTX1 toxins were found in mussels in the same sites, with the OA contents of 0.66 to 6.33 ng/g, DTX1 of N/A to 16.05 ng/g

(Table 1). The OA and DTX1 content of the resin samples were obviously greater than that in mussels, indicating that the SPATT (HP20) could be used as a sensitive monitoring tool for OA and DTXs in the local region. The maximum OA and DTX1 contents in SPATTs (18.10 and 15.04 ng/g) and mussels (6.33 and 16.05 ng/g), harvested at late April and early May 2014, were significantly lower than the international threshold (45 ng OA e.q./g shellfish meat) (Alexander *et al*. 2008). The concentration of homoYTX in mussels (8.55 ~ 22.65 ng/g) was significantly greater than that in SPATTs (1.78~10.00 ng/g), suggesting that HP20 might not be the best resin to absorb the homoYTX. PTX2 and GYM were not quantified and will not be discussed in the present study.

The concentration of OA and DTX1 in water and mussels from the Coast of Gouqi Island was relatively low in the present study, showing that *Mytilus* spp. was clean and safe to public locally during our investigation time. Eight species of mussels, collected at winter of 1999 from close fishing farm (Zhoushan Island), were highly contaminated by OA (15.57~218.95 ng/g) (Yuan *et al.* 2002). Prior to our study, Xu found that the DSP concentration (by ELISA) reached as high as 95.9 ng/g in 122 economical shellfishes in Zhejiang Coast in 2012 (Xu *et al*. 2013). OA and DTX1 were commonly detected over the international threshold recently from Bohai, Yellow Sea, East China Sea and South China Sea from 2010 to 2012 (Lin *et al*. 2015). The application of an effective early warning tool in these farm areas is necessary and urgent.

HP20 was selected to be superior to adsorb the major polyether toxins (MacKenzie *et al*. 2004). The deployment of SPATT (HP20) in Irish waters showed that OA (1200~2640 ng/g), PTX2 (12~220 ng/g), YTX (23~347 ng/g), and up to 617 ng/g of DTX2, 6800 ng/g of AZA1 and 621 ng/g of AZA2 were able to be absorbed and identified (Fux *et al*. 2009). Here, OA, DTX1, PTX2, GYM and homoYTX were detected in HP20. But low absorbent efficiency of homoYTX indicated that the selection of more effective resin for homoYTX requires further investigation.

A time series investigation of the detectable toxins in SPATTs and mussels were displayed in Fig.1. No significant correlation was observed between OA+DTX1 contents in SPATTs and that in mussels. Similarly, Fux *et al*. (2009) investigated the toxins (OA, DTX2, PTX2, AZAs) distributed at the West Coast of Ireland, also showing no significant correlation. Investigations of DA distribution at coastal California (USA) showed that DA in mussels was detected 7~8 weeks later than that in SPATTs during two bloom events (Lane *et al*. 2010). The short term deployment of our SPATT (three months from 11th of April to 4th of July 2014) might be the reason that no clear relationship was found. More samples are collected and further results will be analysed.

Table 1. Toxin profiles and contents detected in SPATTs and mussels from Gouqi Island, Zhejiang Coast along Eastern China.

	Amount of toxin (ng/g)	
Toxin	SPATT	Mussels
OA	1.26-18.10	0.66-6.33
DTX1	0.85-15.04	ND-16.05
homoYTX	1.78-10.00	8.55-22.65
PTX2	Not quantified	Not quantified
GYM	Not quantified	Not quantified

Note: ND stands for not detected.

A clear trend was observed both in SPATTs and mussels that OA+DTX1 toxins were high at April and May, and decreased to low levels in July at all three sampling sites, indicating that spring is the season with the highest risk of DSP outbreaks in Gouqi Island. No clear trend of homoYTX was found in SPATTs and mussels. The seasonal distribution of shellfish toxins was studied along Chinese Coast. Usually, autumn and winter were found to have the highest risk of DSP at low latitude ocean locations (South China Sea) (Hu *et al*. 2013). At high latitude (Yellow Sea and East China Sea), highly DSP contaminated mussels were almost detected in spring (Huang *et al*. 2013). Thus, the deployment of SPATT for DSP should be more frequent and intense in spring and autumn in the East China Sea.

In the present study, SPATT discs (HP20) were deployed at 10-day intervals whereas one-week exposure regimes was used in previous study to validate the efficacy of SPATT (MacKenzie *et al*. 2004; Fux *et al*. 2009; McCarthy *et al*. 2014). The maximal HP20 capacity of OA and DTX1 was reported as 1639 and 2934 μg/g, respectively (Li *et al*. 2011). These values were far greater than our results (<20 ng/g), indicating that our SPATT (HP20) did not reach the saturation point and 10-day deployment was successfully performed.

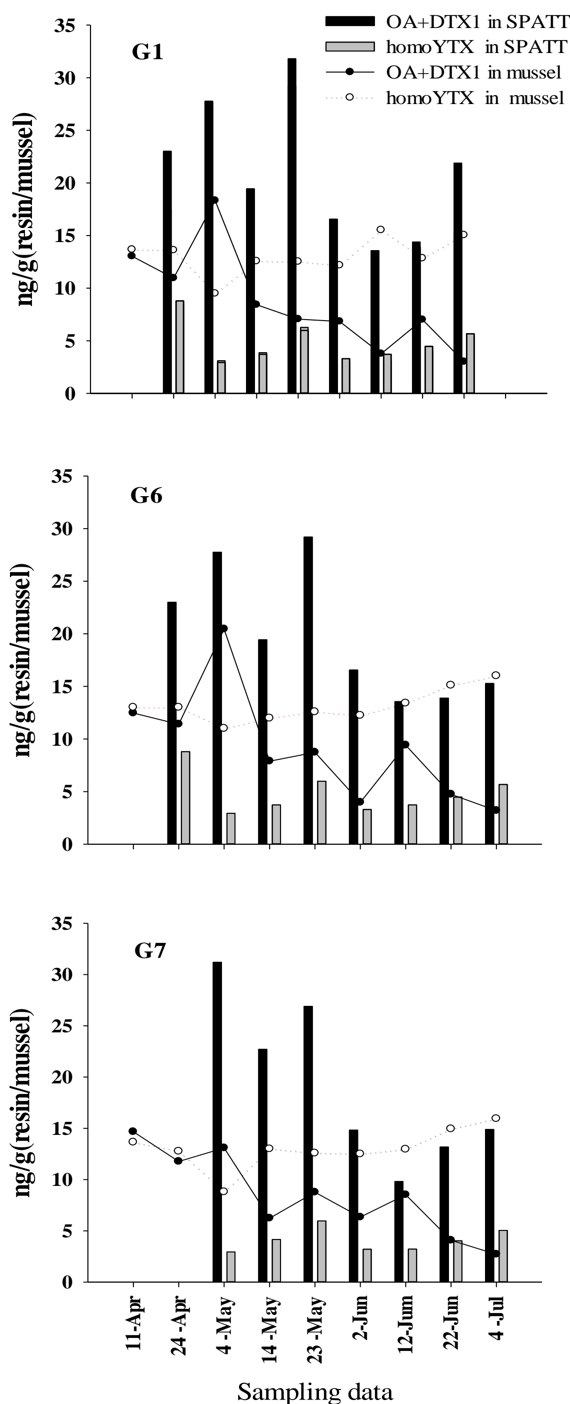

Fig.1. Toxin contents in SPATT and mussel tissues in the site G1, G6 and G7 at the Coast of Gouqi Island from 11 April, 2014 to 4 July, 2014.

In summary, SPATT has the potential to be used as an early warning tool to monitor the occurrence of *Dinophysis* blooms and shellfish contamination events in our investigation area, and further long term studies are required.

Acknowledgements

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Determination of the concentration of dissolved lipophilic algal toxins in seawater using pre-concentration with HP-20 resin and LC-MS/MS detection

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Abstract

Algal toxins are metabolites that can accumulate in fish or shellfish and render these foodstuffs unfit for human consumption. These toxins, released into seawater during algal occurrences, can be monitored through direct analysis of seawater or through passive sampling. Knowledge of the total and dissolved toxin contents is important for different reasons: (i) understanding the ecological role of toxic algae, (ii) modelling of toxin uptake by aquatic organisms and (iii) estimating the risk of toxins in filtered seawater for land-based aquaculture or desalination plants.
Seawater portions of 30 L were collected and pre-concentrated by passive sampling with HP-20 resin over a 48 h period. Detection of lipophilic toxins in the extracts of the resin was carried out using liquid chromatography coupled to tandem mass spectrometry. This combination allowed for the detection of sub-ppb levels of dissolved toxins and will permit future studies to more accurately model the adsorption behaviour of toxins by passive sampling devices.
In particular, we determined the levels of okadaic acid (OA), dinophysistoxin-1 (DTX1), pectenotoxin-2 (PTX2) and pinnatoxin G (PnTX-G) in seawater from Ingril lagoon. Okadaic acid was the most concentrated compound with ca. 8.6 ng L^{-1}, followed by DTX1 with ca. 1.4 ng L^{-1}, and both PTX2 and PnTX-G at ca. 0.2-0.3 ng L^{-1}. To our knowledge this is the first direct analysis of lipophilic dinoflagellate toxins in seawater. However, these concentrations were observed in a lagoon and should be confirmed in open coastal waters.

Keywords: SPATT, passive sampling, marine biotoxins, benthic microalgae, dissolved toxins

Introduction

A decade ago, passive sampling was introduced as a tool to monitor algal toxins (MacKenzie *et al.*, 2004). Since then, a number of field studies have shown the use of the technique to trace an array of algal or cyanobacterial toxins in algal cultures or marine and coastal environments, including hydrophilic toxins such as saxitoxins and domoic acid, and lipophilic toxins, e.g. azaspiracids, brevetoxins, ciguatoxins, microcystins and okadaic acid, (Caillaud *et al.*, 2011; Fux *et al.*, 2009; Fux *et al.*, 2010; Fux *et al.*, 2008; Kudela, 2011; Lane *et al.*, 2010; Shea *et al.*, 2006; Stobo *et al.*, 2008).
In a previous study (Zendong *et al.*, 2014), we have shown the capability of passive samplers to capture toxins from both pelagic and benthic or epiphytic microalgae (Fig. 1). In particular, PnTX-G and DTX1 were accumulated in significant amounts, indicating the presence of both *Vulcanodinium rugosum* and *Prorocentrum lima* in this lagoon.

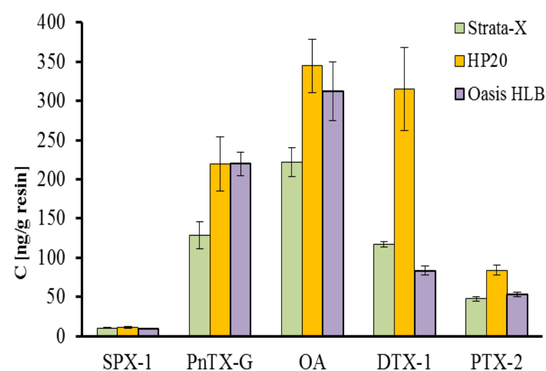

Fig. 1. Accumulation of SPX1, PnTX-G, OA, DTX1 and PTX2 on Oasis HLB, Strata-X and HP-20 SPATTs exposed in July at Ingril Lagoon on a weekly basis (expressed as [ng/g dry resin]).

However, very little is known about the quantitative relationship between the levels of dissolved toxins in seawater and either the levels in shellfish or the levels in the passive samplers. Only a few studies have been able to determine the concentrations of dissolved toxins in the field (Lefebvre *et al*., 2008; Liefer *et al*., 2013; Trainer *et al*., 2009). In particular, no studies were known to us that have determined absolute concentrations of lipophilic toxins in seawater. Thus, we designed an experiment to quantitatively determine the concentrations of lipophilic toxins produced by dinoflagellates in seawater. Our approach consisted in the collection of large volumes of filtered seawater and concentration of the toxins on a lipophilic resin (HP-20), followed by analysis using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Material and Methods

Seawater sampling in Ingril Lagoon

Seawater samples (nine portions of 30 L) were collected from Ingril Lagoon on 2 September 2014. Raw seawater was pumped over two sieves in series of 125 µm and 20 µm phytoplankton mesh using a submersible electric galley pump (GP1352 13 L Whale, USA), operated with FD-7011 batteries (12 V, Lohuis, The Netherlands) into 30 L carboys (polyethylene). A total of nine carboys were filled in this way on the shore. Once in the laboratory, one carboy of filtered seawater was kept separately, while the other eight were combined into a 90 L portion and a 150 L portion. Portions of HP-20 resin (3 g) were prepared in embroidery frames and activated as previously described (Fux *et al*., 2009; Zendong *et al*., 2014). Two, four and six portions of resin were placed into the 30 L, 90 L and 150 L portions of seawater, respectively. The 30 L portion of seawater was stirred using a stir bar with a magnetic stirrer (VCM-C7, VWR, France), while seawater in the two larger containers was circulated using peristaltic pumps equipped with 15 mm flexible plastic tubing (7549-40 Masterflex Cole-Parmer Instrument Co., Chicago, Illinois, USA). Stirring or circulation of seawater was maintained for 48 h before replacing the resin portions with the same number of fresh frames which were deployed for a further 48 h.

Extraction and LC-MS/MS analysis of resins

The portions of HP-20 resin were extracted as previously described (Fux *et a*l., 2009; Zendong *et al*., 2014). All 3g-portions were extracted on separate SPE cartridges for ease of handling. Subsequently, eluates were combined into single fractions for each of the three seawater portions, keeping the extracts from the first and second deployment separate. Thus a total of six final extracts were obtained for LC-MS/MS analysis (three different volumes of seawater each consecutively extracted twice). Final extract volume was kept to 1mL of 50% aqueous methanol for all six fractions.

Analysis was performed on a UHPLC-system (UFLC, Shimadzu) coupled to a triple-quadrupole mass spectrometer (4000Qtrap, ABSciex, Les Ulis, France). Chromatography was performed with a Hyperclone MOS C8 column (50 × 2.0 mm, 3µm) with a C8 guard column (4 × 2.0 mm, 3 µm, Phenomenex). A binary mobile phase was used, phase A (100% aqueous) and phase B (95% aqueous acetonitrile), both containing 2 mM ammonium formate and 50 mM formic acid. The flow rate was 0.2 mL min^{-1} and injection volume was 5 µL. The column and sample temperatures were 25°C and 4°C, respectively.

Two different gradients were employed, one for the negative mode (to analyse OA, DTX1 and dinophysistoxin-2 (DTX2)) starting with 30% B, rising to 95% B over 8 min, held for 2 min, then decreased to 30% B in 0.5 min and held for 4.5 min to equilibrate the system; and a second gradient for the positive mode (for PTX2, 13-desmethyl spirolide C, PnTX-G and pinnatoxin A (PnTX-A) starting with 30% B, rising to 95% B over 2.5 min, held for 5 min, then decreased to 30% B in 0.1 min and held for 2.5 min to equilibrate the system.

Analytes were detected by negative or positive ion mode using multiple reaction monitoring (MRM). The following negative transitions (precursor ion→product ion) were used for quantification and confirmation (confirmatory ions shown in parentheses): OA and DTX2: *m/z* 803.4→255.1 (803.4→113.1); DTX1: m/z 817.5→254.9 (817.5→112.9). Positive ionization transitions were as follows, PTX2: *m/z* 876.6→823.5 (876.6→805.6); 13-desmethyl spirolide C *m/z* 692.6→164.2 (692.6→444.3); PnTX-G *m/z* 694.4→164.1 (694.4→458.3); PnTX-A *m/z* 712.4→164.1 (712.4→458.3).

PnTX-A and -G were quantified against a well characterised standard of PnTX-G from NRCC (Halifax, CA), assuming that PnTX-A had the same response factor as PnTX-G. All other toxins

were quantified against certified calibrants (NRCC, Canada).
The ESI interface was operated using the following parameters, in negative mode: curtain gas 20 psi, temperature: 550°C, gas1: 40 psi; gas2: 50 psi, ion spray voltage: -4500 V; in positive mode: curtain gas: 30 psi, temperature: 450°C, gas1: 50 psi; gas2: 50 psi, ion spray voltage: 5500 V.

Results and Discussion

Four toxins were quantifiable in at least one of the portions of seawater: OA, DTX1, PTX2 and PnTX-G. Traces of 13-desmethyl spirolide C were also detected. This profile is coherent with our previous results obtained with passive sampling directly in this lagoon (Zendong et al., 2014). Okadaic acid was the most concentrated toxin, with a maximum of ca. 6.9 ng L^{-1} determined in the first extraction (S1-30L) of the 30 L portion (Table 1). The second extraction (S2, Table 1) yielded typically around 20% of the cumulative total for all toxins except DTX1, for which slightly higher proportions were detected in the second extraction. The other toxins were less abundant but all were above limit of quantification (LOQ), except PTX2 in the second extraction of the two larger seawater portions. The highest LOQs were those for OA and DTX1, i.e. both ca. 5 ng mL^{-1} of injected solution (or ca. 0.2 ng L^{-1} seawater), and the lowest LOQ was the one for PnTX-G, i.e. 0.5 ng mL^{-1} injected solution (or ca. 0.02 ng L^{-1} seawater).

The concentrations of all of the toxins were lower in the larger water portions, typically by ca. 50%, than those in the 30 L portion. We believe that this is due to an artefact of adsorption of the dissolved toxins to the container wall of the polyethylene carboys, and subsequent loss when the 30 L portions were combined into the larger containers of 90 and 150 L, respectively. The fact that both the 90 L and the 150 L portion yielded the same toxin concentration also suggests that container adsorption may be a dominant factor in the losses observed.
Therefore, future exercises should attempt extraction of the water portions directly in the sampling container, as was done with the 30 L portion in this experiment. Seawater collection in transportable containers of 20 to 30 L is practical and as several portions were deployed in each of the containers anyhow, and extracted separately to pre-concentrate the toxins from seawater, such a procedure would not constitute a major obstacle.

Table 1. Concentrations of toxins in seawater of Ingril Lagoon, September 2nd 2014 (in [ng L^{-1} of seawater]);

Sample	OA	DTX1	PTX2	PnTX-G
S1-30L	6.9	1.0	0.2	0.2
S1-90L	3.5	0.7	0.1	0.1
S1-150L	3.5	0.9	0.1	0.1
S2-30L	1.7	0.4	0.1	0.05
S2-90L	1.1	0.5	*n.d*	0.02
S2-150L	0.9	0.5	*n.d*	0.02
Total-30L	8.6	1.4	0.3	0.25
Total-90L	4.6	1.2	0.1	0.12
Total-150L	4.4	1.4	0.1	0.12

S1-volume refers to the seawater concentration as determined by the first resin portion (T_0 to 48 h), S2-volume refers to the seawater concentration as determined by the second resin portion (48 h – 96 h); n.d. = not detected.

The fact that 20% of the toxins were still recovered in the second extraction suggests that both adsorption dynamics and kinetics may play a significant role for this experimental design. This is consistent with the observations mentioned above for the losses in transferring the seawater from several 30 L carboys to larger containers. As previously shown, salinity also appears to affect adsorption kinetics of lipophilic toxins on HP-20 resin (Fan et al., 2014). Seawater salinity at Ingril Lagoon during the present study was quite elevated (ca. 40). Therefore, future studies should also take this factor into consideration.

This experiment has overcome the typical limitations of classical solid-phase extraction (SPE) of seawater which is known to suffer from break-through phenomena for volumes greater than 1 L.

Overall, these results are encouraging to apply a similar design to the analysis of coastal seawater from open bays, since the limits of quantitation were surpassed by ca. 10 to 15-fold in the first extraction, even for the smallest volume of seawater used here (30 L).

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Mitigating fish-killing algal blooms: clay revisited to remove ichthyotoxins

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Abstract

Previously we demonstrated effective removal of *Prymnesium parvum* toxicity towards the gill cell line RTgill-W1 by Phoslock™ clay and have since worked on refining clay type and dosage. In this work we explored 12 commercially available Australian clays (two zeolites, four kaolins, six bentonites) and two types of Korean yellow loess for removal of *Prymnesium* toxicity towards the gill cells under differing pH conditions. At pH 7, gill cell viability improved slightly after treatment with kaolins and zeolites (22-26% viability increase), with bentonite clays and Phoslock™ performing best (up to 57% viability increase). Korean loess proved unsuitable for removing ichthyotoxins. However, at pH 9, kaolin, loess and zeolite exacerbated ichthyotoxicity and toxin removal by Phoslock™ was significantly reduced (9% viability increase vs. 57% at pH 7). Interestingly, bentonites completely removed *Prymnesium* ichthyotoxicity at pH 9, thus suggesting their potential as rapid response tools during high density (high pH) fish-killing algal bloom events. Additional screening against other fish-killing algae revealed that bentonite clay could only partly remove *Alexandrium catenella* and *Chattonella marina* ichthyotoxins, whereas complete removal of *P. parvum* and *Karlodinium veneficum* toxicity was achieved at clay loadings between 0.05-0.25 g L^{-1}.

Keywords: mitigation, ichthyotoxin, clay, adsorption, pH, RTgill-W1

Introduction

Previous strategies to mitigate ichthyotoxic effects of harmful algal blooms have mainly focused on removing cells by flocculation or by destruction of the causative species through cell lysis via chemical, UV or sonication treatments. These latter approaches risk disruption of algal cell membrane integrity, which can amplify fish-killing effects through the release of intracellular ichthyotoxins (Deeds *et al*., 2002). A more promising approach to mitigation is the flocculation of harmful algal cells through the application of clay, a major natural constituent of marine sediments. Successfully employed by the South Korean government since 1996, after an extensive *Cochlodinium polykrikoides* bloom caused the loss of USD$96 M worth of caged yellowtail sea bream (Park *et al*. 2013), researchers have focused on optimising cell flocculation. Screening of several different clay types against a variety of algal species revealed cell removal efficiency to be dependent upon algal species and clay type (Sengco and Anderson, 2004). Later work indicated that not only algal cells, but also dissolved, extracellular ichthyotoxins can be removed through clay application. This property of clay has so far only been demonstrated for bentonite based clays in the case of brevetoxins (Pierce *et al*., 2004), microcystins (Prochazka *et al*. 2013) and *Prymnesium parvum* ichthyotoxins (Seger *et al*., 2014, Sengco *et al*., 2005). The potential of other clay types to remove ichthyotoxins and the environmental parameters governing toxin adsorption currently remain unknown. In our studies, we initially achieved up to 100% removal of *P. parvum* toxicity towards the gill cell line RTgill-W1 using a specialised, bentonite based clay designed for phosphate removal purposes (Phoslock™; Seger *et al*., 2014), but in later trials failed to significantly reduce such ichthyotoxicity

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in high density *Prymnesium* cultures at pH above 9.

We here explored the ability of 12 commercially available Australian clays and two types of Korean yellow loess to reduce toxicity of *P. parvum* preparations towards the gill cell line RTgill-W1 at pH 7 and 9 to interpret our previous observations. The most effective clay type and dosage was determined and the potential of clay to adsorb ichthyotoxins released from lysed *Karlodinium veneficum, Chattonella marina* and *Alexandrium catenella* preparations assessed.

Material and Methods

Prymnesium parvum (UTAS culture code PPDW02), *Chattonella marina* (CMPL01), *Karlodinium veneficum* (KVSR01) and *Alexandrium catenella* (AC.CH.02) were grown in 500 mL conical glass flasks containing 300 mL of GSe medium (L1 for AC.CH.02) at 20°C (17°C for AC.CH02), salinity of 30 psu and 120 µmol photons m^{-2} s^{-1} of light. *Prymnesium* cultures for pH manipulation experiments were concentrated through centrifugation, lysed through sonication and diluted in L-15/ex medium (Schirmer *et al*., 1997) with pH adjusted to either 7 or 9 (0.1M HCl or NaOH) to yield a concentration equivalent to 10^5 cells mL^{-1}. Preparations were then treated with the different clay types (1 g L^{-1} final concentration; Table 1), vortexed for 5 s and centrifuged. The supernatant was tested on the gill cell line Rtgill-W1 in conventional 96-well plates (as per Dorantes-Aranda et al., 2011) and the most appropriate clay type for removal of toxicity towards the gill cells determined. Following the same protocol as described for *P. parvum*, this clay (Ed bentonite) was applied to sonicated preparations of the other algal species (0-0.25 g L^{-1} final concentration) and the supernatant tested on the gill cells in specialised microplates with inserts (Corning 3381; Dorantes-Aranda *et al*. 2011). As an indicator of gill cell viability, cellular metabolic reduction of the dye Resazurin to fluorescent Resorufin was quantified in a plate reader (BMG Labtech) and reported as % viability compared to a non-toxic control (seawater + clay).

Results

The effect of clay treated *P. parvum* preparations on gill cell viability was strongly dependent on clay type and pH (Fig. 1). Unmodified clays could be grouped according to their major clay mineral component based on comparable effects on gill cell viability, whereas the modified clays (B1-AM and Phoslock™), as well as weathered bentonite (Es) did not conform to any core clay mineral group (Table 1; Fig. 1.). At pH 7, no significant negative effect of clay treatment on the gill cells was observed and most clay groups increased gill viability significantly compared to the untreated, toxic control (only 20% viable gill cells remained after 3.5 h exposure; no significant difference in toxicity between pH 7 and 9). The bentonite group and Phoslock™ performed best (+54 and +57% viability compared to toxic control), followed by Es (+38%), Kaolins (+26%) and Zeolites (+22%). Korean loess and B1-AM did not influence gill cell viability at pH 7. However, at pH 9, the increase in gill cell viability previously observed for Phoslock™ and Es clays at pH 7 was reduced (only ~10% viability increase) and toxicity exacerbated by application of B1-AM (-9%), kaolin (-11%), Korean loess (-15%) and zeolite (-17%). Interestingly, at pH 9, the performance of the unmodified bentonite group was improved, resulting in complete removal of *P. parvum* toxicity towards the gill cells.

Table 1. Grouping of different commercially available clays screened in this study based on similarities in ichthyotoxin adsorption (see results section).

Clay grouping	Commercial name	Description
Korean loess	Natural loess from Tongyeong and Geoje	Kindly provided by Tae-Gyu Park
B1-AM	B1-AM	50% bentonite and 50% gypsum
Zeolites	ANZ38, Escott	Unmodified
Kaolins	CA-1, Kaolinite (Zeolite Australia), Snobrite C, Ceramic	Unmodified, ceramic clay sourced from local potterer
Es	Es	Weathered bentonite
Phoslock	Phoslock™	Modified with Lanthanum cations (5%)
Bentonites	A, Ed, Trubond	Unmodified

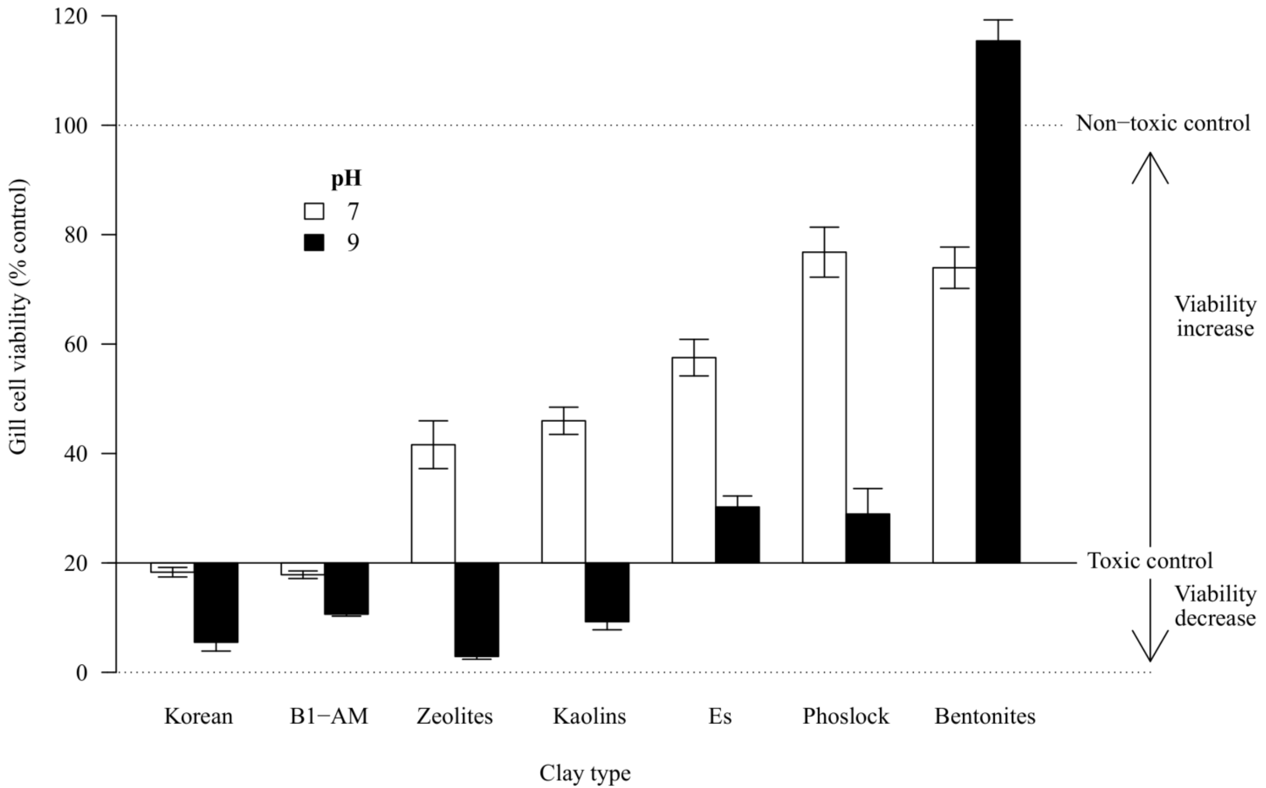

Fig. 1. Gill cell viability after 3.5 h exposure to lysed *P. parvum* preparations at pH 7 and 9 (white and black bars, respectively) treated with different clay types (1 g L^{-1}). Arrows indicate direction of viability change in relation to toxic control (solid black line). Error bars represent the standard error (n=3).

Further testing of a representative clay of this unmodified bentonite group (Ed) on lysed, undiluted *P. parvum, Karlodinium veneficum, Chattonella marina* and *A. catenella* cultures to determine minimum effective dosages revealed significant differences in the clay's efficacy between the different ichthyotoxic algae (Figure 2). Viability of gill cells exposed to *P. parvum* and *K. veneficum* cultures increased hyperbolically in response to treatment with increasing clay concentration. Complete removal of toxicity occurred between 0.05-0.1 g L^{-1} in case of *P. parvum* and between 0.1-0.25 g L^{-1} for *K. veneficum*. However, treatment of *C. marina* and *A. catenella* cultures with Ed clay only increased gill viability up to a maximum of +20 and +15%, respectively at a clay dosage of 0.1 g L^{-1}. Higher clay concentrations did not result in any further viability increases.

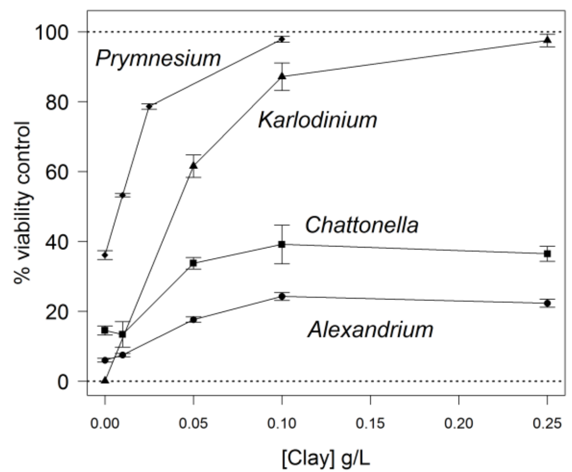

Fig. 2. Gill cell viability after 2 h exposure to lysed *P. parvum*, *K. veneficum*, *C. marina* and *A. catenella* cultures treated with bentonite clay (Ed, 0-0.25 g L^{-1}). Error bars represent the standard error (n=3).

Discussion

Mitigation of ichthyotoxic algal blooms through clay flocculation has been widely explored, yet the capacity of clay minerals to adsorb dissolved ichthyotoxins has received little attention. Through treatment of potent ichthyotoxic algal preparations with clay, we were able to significantly reduce and in the case of *P. parvum* and *K. veneficum,* completely eliminate damage to the gill cell line RTgill-W1. These toxin adsorptive characteristics of clay have previously only been described for bentonite type clays (Sengco *et al*., 2005, Pierce *et al*., 2004, Prochazka *et al*., 2013, Seger *et al*., 2014), but in the case of *P. parvum* ichthyotoxins also extend to clays of the zeolite and kaolin group, albeit only effective at pH 7. The minimum effective dosages reported here for *P. parvum* should be interpreted with care, since we have recently discovered evidence of adherence of *P. parvum* ichthyotoxins to the multi-well plates employed in this study (Dorantes-Aranda *et al*., ICHA 16 Proc). Nonetheless, we have gained vital insight into the role pH plays in ichthyotoxin adsorption. Significantly decreased ichthyotoxin adsorption at higher pH by most clay types, including Phoslock™, explains our previous failure to reduce toxicity in dense (10^6 cells mL^{-1}, pH 10) *P. parvum* cultures. Our extensive clay screening revealed that at elevated pH associated with dense algal blooms, bentonite type clays are the best suited for ichthyotoxin removal purposes. However, the extent of ichthyotoxin adsorption and the amount of bentonite clay required to completely eliminate ichthyotoxicity differs between algal species. The fact that bentonite clay was only able to remove part of the toxicity of *A. catenella* and *C. marina* towards the gill cell line highlights the complexity of the ichthyotoxic principles at work and suggests the presence of distinctly different ichthyotoxic compounds by these different fish-killing HAB species. While karlotoxins could be efficiently removed by clays, almost certainly due to their amphipathic nature (Wagoner *et al*. 2008), the unknown ichthyotoxic exudate(s) produced by *Alexandrium* (Ma *et al*. 2011), were not efficiently removed. Adsorption of only a fraction of the ichthyotoxicity points to the presence of multiple chemical compounds. In case of the reactive oxygen species (ROS) mediated ichthyotoxicity of *C. marina* (Marshall et al. 2003), this suggests preferential adsorption of selected lipid peroxidation products and/or ROS.

We have shown that the potential of clays to be employed as mitigation agents of fish-killing algal blooms extends beyond their cell flocculating properties, with ichthyotoxin adsorption representing an important additional benefit to clay application. We are now seeking to further characterise the physical properties (zeta potential, chemical composition) of the clays employed in this study to define the desirable properties of local clays. A more detailed discussion of these results will be published elsewhere (Seger et al. 2015). Further work will focus on assessing the potential of clay to remove ichthyotoxins from other harmful species, such as *Karenia mikimotoi*, *K. brevis*, *Amphidinium carterae* and *Heterosigma akashiwo.*

Acknowledgements

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Hydraulics and barley straw (*Hordeum vulgare*) as effective treatment options for a cyanotoxin-impacted lake

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Abstract

Many nutrient-rich freshwater systems are dominated by summer blooms of cyanobacteria, some toxic. In Lake Williston, a recreational lake in Maryland, USA, summer blooms of toxic *Microcystis aeruginosa* have become a major threat to use of the lake due to microcystin concentrations exceeding 2 ppm (2 mg L^{-1}). Bloom mitigation was attempted through several techniques including flocculation, various chemical treatments, winter hydraulic flushing, and deployment of barley straw (*Hordeum vulgare*). The latter 2 interventions, reported below, were effective in limiting bloom formation and toxin accumulation, with flushing apparently removing large portions of over-wintering *M. aeruginosa* populations needed for re-inoculating surface assemblages and spring barely straw deployment limiting subsequent summer growth of any remnant populations.

Keywords: Microcystis blooms, mitigation, flushing, barley straw

Introduction

Cyanobacteria blooms are increasing throughout the world due to human-derived nutrient enrichment of fresh and tidal-fresh aquatic systems, combined with increasing temperatures associated with climate change. In eastern Maryland, USA, Lake Williston is a 65 acre recreational lake (464,297 m^3 estimated volume based on bathymetric measurements) surrounded by poultry-litter fertilized croplands, leading to excessive nitrogen and phosphorus in soils and surface waters, and for nitrogen, groundwater. Runoff and base flow from groundwater, as well as atmospheric deposition of ammonia-N immediately after litter application (18 µM, unpubl. data), result in spring nitrate-N and phosphate-P levels exceeding 165 and 3.3 µM, respectively (N/P=50). For Lake Williston with minimal discharge and thermal stratification, the nutrients support extensive summer cyanobacteria blooms, dominated by toxic *Microcystis aeruginosa*. Microcystin levels in late summer are substantial, exceeding WHO (and Maryland) guidelines for drinking water (1 µg L^{-1}) and recreational use (10 µg L^{-1}), resulting in lake closure, a major loss for the lake's use as a summer camp for girl scouts from the region. In an attempt to assist camp managers in re-opening the camp for summer use, several methods were employed to eliminate or reduce the blooms and accumulating toxins, including winter lake flushing to remove overwintering *M. aeruginosa* populations, and spring deployment of barley straw *Hordeum vulgare*.

Material and Methods

Lake Williston is located ~10 km from Denton, MD, USA (38°49'51.2"N, 75°50'26.92"W). Nutrient concentrations were measured in two feeder streams and the lake, with concentrations determined using standard nutrient procedures (Lawrence *et al*. 1995). Twice monthly samples were also collected to determine species composition of the autotrophic plankton, microcystin levels, and using a Turner Designs Aquafluor®, fluorescences of chlorophyll *a* (chl) and phycocyanin (PC). Additionally, an *in water* continuous sensor was deployed in the lake near

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the scout camp (~3 m depth) to collect chlorophyll, DO, and pH levels.

Lake Williston water depth is controlled by a manually-operated dam; winter lake volumes were reduced by removing portions of the dam to allow 80% of the lake to drain in ~8 h, flushing out over-wintering plankton assemblages and exposing the lake flanks to winter air temperatures. The dam was reset in March, allowing the lake to refill. Thereafter, 500 bales (@approx. 0.5 m x 0.5 m x 1 m) of *H. vulgare* were deployed around the feeder streams and lake. Shallow shore sediment cores were collected in May 2012 and returned to the laboratory where water over the core was transferred to 50 mL tubes. After removing the remaining water above the core, 50 mL of filtered lake water was added to tubes and cores and placed on a window sill. After 19 d, both tubes and cores were moved to a 10:14 L:D fluorescently-lit incubator and the temperature gradually raised over 12-15 d to 28.5°C when chl and PC fluorescences were determined and samples removed for species determination.

Results and Discussion

In 2010 and 2011, prior to flushing and barley straw deployments, summer (Jun-Aug) *Microcystis* abundances and microcystin concentrations were high, exceeding 10^6 mL^{-1} and 80 ppb, respectively (Fig. 1). These elevated levels

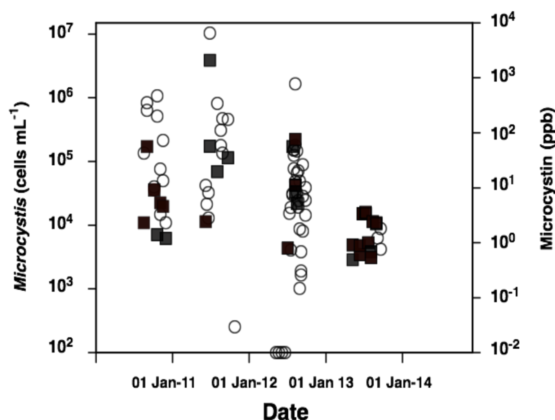

Fig. 1. *Microcystis* (cells mL^{-1}, o) and microcystin (µg L^{-1}, ■) from 2010-2013 in Lake Williston. Note the logarithmic scales.

were noted with almost undetectable NO_3-N and DIP-P, suggesting likely sufficient access to each nutrient through continuous baseflow (regional groundwater input >8-10 mgL^{-1}, K. Staver, pers. comm.) for N and rapid recycling for both N and P. Indeed, nutrient flux from lake sediments would be very high due to the fluxes that accompany lake pH >9.2 (J. Crornwell, pers. comm., Seitzinger 1991), routinely exceeded during the summer cyanobacteria maxima and CO_2 drawdown in photosynthesis (Fig. 2).

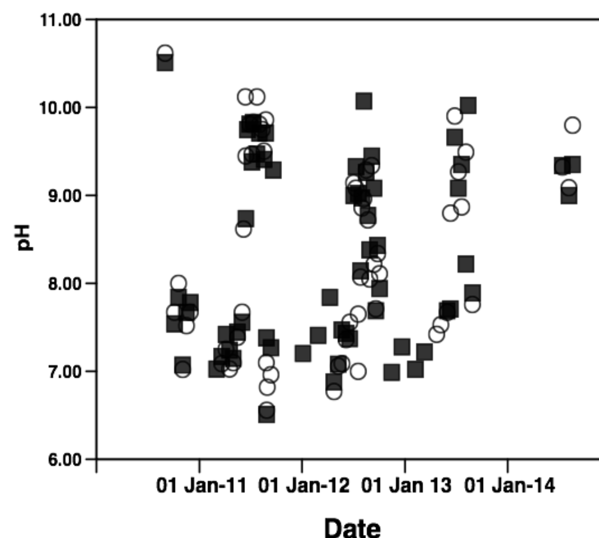

Fig. 2. pH over time at the Beach (■) and dam (o) stations of Lake Williston, 2010-2013.

In the summers of 2012 and 2013, however, both *Microcystis* abundances and microcystin levels were lower than noted in the summers of the 2 previous years (Fig. 1): all but 1 sample had Microcystis at $\leq 10^5$ mL^{-1} and microcystin declined to <6 ppb in 2013. This dramatic reduction in the cyanobacterium and hepatotoxin is attributable to 2 treatments employed in the lake, winter drawdown and spring barley straw deployment. As reported previously (e.g., Preston *et al.* 1980), *M. aeruginosa* overwinters by settling to bottom sediments where on resuspension the following year (e.g., Verspagen *et al.* 2004), it may encounter abundant nutrients and warm temperatures favoring growth and bloom development (sheets of the cyanobacterium were kicked up in an early spring wade-in in 2010, K. Sellner, pers. observation). The draining of the lake over the 2011 and 2012 winters decreased total volume from 4.64 x 10^5 m^3 to 0.9 x 10^5 m^3 in 8 h, yielding an average discharge rate of 12.7 m^3 s^{-1}, rapidly exposing the littoral zones of the lake and through bottom shear stress, resuspending settled, overwintering *Microcystis* populations and funneling them towards/to the dam for settling in the deeper main basin with

much lower resuspension potential and hence low re-inoculation for future blooms (Brunberg and Blomqvist 2003) or discharging into the stream immediately downstream of the lake. Additionally, the drained lake littoral zones were exposed to winter temperatures, favoring ice crystal formation and cell lysis in any *Microcystis* remaining in the shallow sediments after lake draining. The possible role for this process in limiting re-introduction of *Microcystis* populations was examined in the core experiments. Cyanobacteria and other eucaryotes did emerge from both the overlying water incubated from above the cores as well as the cores themselves, and included *Geitlerinema acutissimum*, *Pseudanabaena* sp., and *Synechococcus*; however, *M. aeruginosa* was not present in the final samples. Although these results are supportive of littoral zone exposure to freezing temperatures in limiting *Microcystis* survival, it is acknowledged that 1) lake draining could have removed all populations to deeper portions of the lake or over the dam or that 2) the incubation procedure may not have supported viability in the cyanobacterium.

The other treatment limiting reappearance of *Microcystis* and microcystin was the lengthy exposure to barley straw. Decomposition of the straw yields phenolic compounds that retard cyanobacteria growth (Rajabi *et al*. 2009, Waybright *et al*. 2011), but only when the straw is deployed weeks-to-a-month prior to bloom development. Barley straw has been effective in controlling cyanobacteria growth in many freshwater and brackish systems for more than 3 decades (see references in Brownlee *et al*. 2003). Locally, it has been deployed in a small sediment pond at a nearby farm for several years, eliminating all cyanobacteria. Further, water from the pond dramatically depressed the growth of *M. aeruginosa* LE-3 (Fig. 3). More recently, barley straw bales have also been distributed in a *Microcystis*-dominated dredge material lagoon in mesohaline Chesapeake Bay, with lower and later appearances of the cyanobacterium after deployment (Sellner *et al*., in prep.). Although natural decomposition of the straw in waters of the lake and tributary streams appeared to aid in reducing populations of the cyanobacterium and its associated toxin, microcystin (Fig. 1), future mitigation might be even more effective by enhancing decomposition of the lignin-rich material, as more rapid and larger amounts of limiting phenolic residues would be produced. In some preliminary experiments, growth inhibition of *M. aeruginosa* LE-3 was 10-fold greater after exposure to extracts of barley straw bales inoculated with white-rot fungi (*Trametes versicolor* and *Ceriporiopsis subvermispora*)

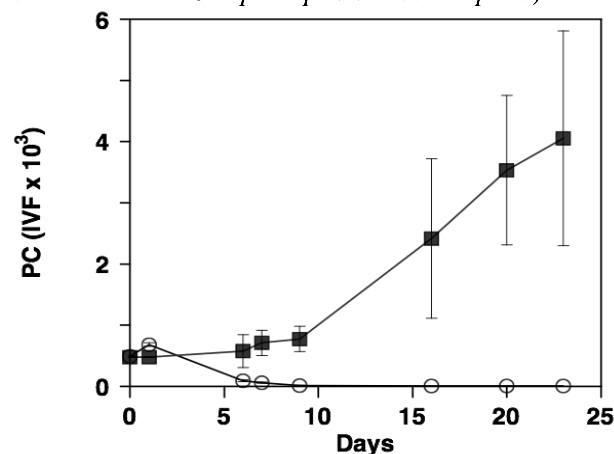

Fig. 3. Growth suppression of *M. aeruginosa* LE-3 on exposure to water from local barley straw treated pond water (McConnell farm, o) vs. water from barley straw-free Lake Williston (■). Vertical bars represent s.e.

known to break down straw vs. inhibition noted for non-inoculated straw (data not shown); the inhibition was substantially greater when the inoculated bales were deployed in the light rather than the dark (Fig. 4), indicating the importance of light-mediated decomposition. Field experiments on effectiveness and estimating costs for lake-wide application and fungal additions are planned for the near future.

In summary, a nutrient-rich, warm lake in eastern MD, USA has been characterized by large toxic blooms of *Microcystis aeruginosa* and microcystin concentrations exceeding 80 ppb in 2010 and 2011, closing the lake for use by local citizens and campers. Levels of cells and toxin have been substantially reduced allowing recreational use of the lake following implementation of two strategies, rapid winter flushing of the lake to reduce its volume to 20% of its size in 8 h and ex- posing the shallow littoral zones to winter freezes and spring deployment of barley straw *Hordeum vulgare*. There are several critical factors to consider in adapting these two strategies in other systems: 1) Lake flushing must be rapid to generate sufficient bottom shear to suspend overwintering *Microcystis* populations from the bottom. This is exemplified by a recent change in dam engineering which now slows

draining to 2 weeks instead of 8 h and has reduced its effectiveness.

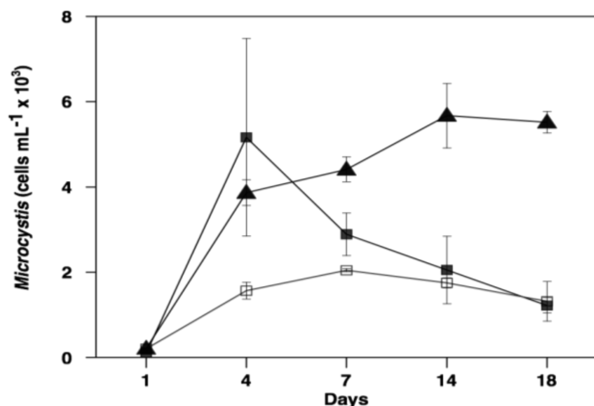

Fig. 4. Growth depression in *M. aeruginosa* LE-3 after exposure to 0.01% (v/v) fungal-enriched barley straw extract from the field. The extract was from barley bales in the field under light (full sun, □) and dark (■) exposures; growth in control, non-fungal enriched extracts depicted with ▲. Vertical bars represent s.e. of the mean.

Although other factors were also likely in the return of large summer blooms of the cyanobacterium after the new dam's construction (e.g., delayed barley straw deployment), high discharge/flushing rates must be ensured to re-suspend the bottom-associated *Microcystis* populations for potential advection to the deep lake basin or discharge over the dam. Slow bottom currents will not be effective in dislodging and transporting the cyanobacterium, and hence 'seed' populations will remain for re-inoculating blooms. 2) Spring deployment of barley straw bales must occur to allow accumulation of decomposition products prior to any accumulation of the cyanobacterium in euphotic depths. Once a bloom is in place, barley straw additions will be ineffective. 3) More rapid decomposition of barley straw, as would occur with white rot fungi additions to the straw, would increase barley straw inhibition of *Microcystis*. Hence, use of fungi-inoculated bales or avoiding fungicide-treated barley straw could lessen the emergence of the bloom-forming cyanobacteria. 4) Although the data are not presented, old (>1 yr) barley straw is less effective in inhibiting the cyanobacterium. And 5) to ensure local acceptance and participation rather than objection to bloom intervention, local citizens must be engaged from the start of any discussions of blooms, toxins, their impacts and risks, and possible mitigation procedures (VanDolah *et al*. 2014).

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Maximising the value of tropical micro-algae in the Cawthron Institute Culture Collection of Micro-algae

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Abstract

Micro-algae maintained in the Cawthron Institute Culture Collection of Micro-algae (CICCM) underpin research globally. One goal when adding new species to the collection is the reliable sourcing of target compounds, e.g. purified standards for chemical analyses and toxicity testing. Suitable isolates from environmental samples are fully characterized to species level using molecular tools and chemical tests and the body of information is attached to each strain. Recent research at the Cawthron Institute has focused on tropical isolates from the Pacific, and the CICCM now maintains species in the genera *Gambierdiscus* and *Fukuyoa* that produce maitotoxin (MTX), putative MTX-3 and/or ciguatoxin (CTX). These isolates are mass cultured for production of ciguatera fish poisoning (CFP) toxins. Species in the genus *Ostreopsis* produce palytoxin (Pltx)-like compounds, which may become a regulated marine toxin. If regulation is imposed, toxin standards are available and rapid test methods (LC-MS/MS) have been developed at the Cawthron Institute using material derived from cultures in the CICCM.

Keywords: Culture collection, micro-algae, dinoflagellates, *Ostreopsis*, *Gambierdiscus.*

Introduction

The Cawthron culture collection originally grew out of personal collections, built up prior to 1990 by Drs Lincoln MacKenzie and Lesley Rhodes. These individual collections were vital for enabling research into harmful algal blooms with regard to seafood safety, but culture maintenance absorbed a great deal of time. A contract was negotiated with the New Zealand Foundation for Research, Science and Technology (FRST) in 1995-6, which specifically allowed for "the provision of a national collection of toxic and noxious microalgae as a reference and resource for the aquaculture industry, researchers and the public health sector". An alarmed, light and temperature controlled facility was then built at the Cawthron Institute. A FRST Science Area Review of Biotoxins was carried out in 1996 and the panel decided that the collection was "an extremely valuable national resource and its continued existence and development needs to be secured". They further recommended that the collection be registered as a National Data Base and designated the collection (18 October 1996) as a "nationally significant database and collection". The CICCM has been fully curated since, and >450 strains of micro-algae are maintained (Fig. 1), either as living or cryopreserved cultures.

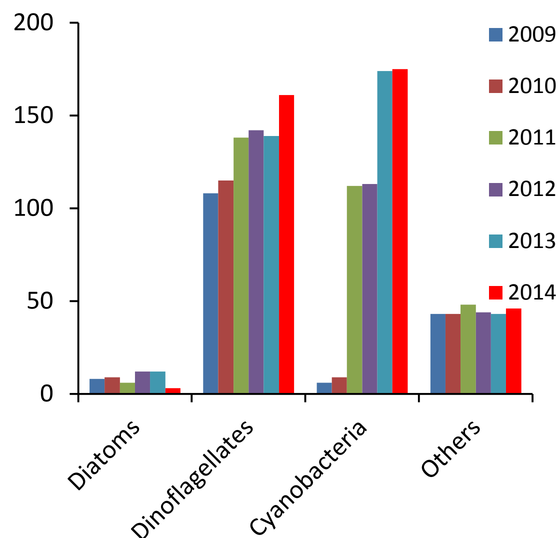

Fig. 1. The numbers of micro-algae isolates maintained in the Cawthron Institute Culture Collection of Micro-algae over the last 6 years.

Current research at the Cawthron Institute (carried out through the Safe New Zealand Seafood programme and funded by the New Zealand government, Contract No. CAWX1317) includes the determination of the risks of CFP and Pltx poisoning from the consumption of contaminated fish or shellfish. To this end, *Gambierdiscus, Fukuyoa* and *Ostreopsis*, and key co-occurring

dinoflagellate species, have been isolated from New Zealand's mainland, the Kermadec Islands group, Hawaii, New Caledonia and the Cook Islands. The isolates, which are maintained in the CICCM, have been characterised using molecular tools, including qPCR and DNA sequence data analysis, and chemical tests (LC-MS/MS). Toxicity studies have also been carried out (see Rhodes *et al.* in these proceedings).

Material and Methods

The living cultures in the CICCM are regularly sub-cultured into growth media as per Protocol CICCM Procedures Manual (Issue No. 4, 20/08/12). At the present time, identification is mainly by DNA sequence data analysis, although in the past cultures were identified based on their morphology, using light and electron microscopy. The optimal conditions for growth and/or toxin production are determined during the research phase and provided to end-users. Whenever possible, cryopreservation is carried out, as described previously (Giménez Papiol *et al.* 2013; Rhodes *et al.* 2006; Wood *et el.* 2008), and banking of new isolates under liquid nitrogen is regularly conducted. This cryopreserved collection comprises mainly cyanobacteria and small flagellates, but also small dinoflagellates, for example, *Amphidinium carterae* CAWD152 from the tropical collection. The cryopreservation Dewars and the living collection are held in alarmed, temperature controlled and monitored rooms and cabinets.
Chemical analyses for MTX, CTX and Pltx were carried out as described previously (Rhodes *et al.* 2014a; Selwood *et al.* 2012).

Results and Discussion

The CICCM is the only one of its kind in New Zealand, and is highly ranked in the Asia-Pacific region, where it is a member of the Network of Asia Oceania Algae Culture Collections (http://mcc.nies.go.jp/AOACC/Home).
Tropical and sub-tropical dinoflagellate genera lodged in the CICCM during research into the causes of CFP and Pltx poisoning, include *Gambierdiscus, Fukuyoa, Ostreopsis, Coolia, Prorocentrum* and *Amphidinium* (Table 1; refer http://cultures.cawthron.org.nz).
Fukuyoa paulensis (CAWD210, 211) was previously named *G.* cf *yasumotoi*, but was recently reclassified (Gomez *et al.* 2015). It was isolated from the sub-tropical coastal waters of northern New Zealand and produces putative MTX-3, as do strains of *G. pacificus* (CAWD213, 227) from the lagoons surrounding Rarotonga, Cook Islands. *G. australes* (CAWD149), also from Rarotonga, produces MTX, while an isolate of *G. polynesiensis* (CAWD212) produces CTX (Rhodes *et al.* 2013; Rhodes *et al.* 2014a).
Two genetically similar *Ostreopsis* species were isolated from the Cook Islands (CAWD184) and the Kermadec Islands (CAWD221); the former is described as *Ostreopsis* sp. 3 by Sato *et al.* (2011). The Cook Islands isolate was non-toxic whereas CAWD221 produced Pltx-like compounds (Rhodes *et al.* 2014b). Characterisation of these isolates is on-going.

Table 1. Sub-tropical and tropical dinoflagellate species in the genera *Amphidinium, Coolia, Fukuyoa, Gambierdiscus, Ostreopsis* and *Prorocentrum* maintained in the Cawthron Institute Culture Collection of Micro-algae (CICCM). Isolates were from Northland, New Zealand, the Kermadec Islands, Hawaii and the Cook Islands.

Species	CICCM code	Toxins
A. cf. *boggayum*	CAWD164	-
A. carterae	CAWD22, 23, 57, 152	-
A. massartii	CAWD132, 156	-
A. mootonorum	CAWD161	-
A. operculatum	CAWD156	
A. trulla	CAWD68	-
C. malayensis	CAWD77, 98, 151, 175, 214*	*
G. australes	CAWD149	MTX
G. pacificus	CAWD213, 227	MTX; Putative MTX-3
G. polynesiensis	CAWD212	CTX
F. paulensis	CAWD210, 211	MTX; Putative MTX-3
O. siamensis	CAWD96, 147, 206, 208	Pltx
O. ovata	CAWD174	Pltx
Ostreopsis sp. 3[#]	CAWD184[#], 221	NTD; Pltx
Ostreopsis sp.	CAWD185	-
P. lima	CAWD33, 6970, 94, 95, 157, 176, 189	OA
P. maculosum	CAWD158	NTD
P. rhathymum	CAWD226	NTD

MTX: maitotoxin; CTX: ciguatoxin; Pltx: palytoxin-like compounds; OA: okadaic acid; NTD: no toxin detected; -: not tested; *toxic by i.p. injection of mice, Rhodes *et al.* 2014c; [#]: Sato *et al.* 2011.

O. siamensis is a common epiphytic bloom former in New Zealand's northern waters, although it has been isolated as far south as the temperate

Marlborough Sounds. Regulation of Pltx in shellfish is likely to occur in the future, due to fears of human illnesses in the Mediterranean, and the toxic isolates in the CICCM have been the basis for development of rapid chemical test methods, which employ the oxidative cleavage of marker fragments (LC-MS/MS; Selwood *et al.* 2012). Research into the much-neglected health issue of CFP is on-going and the cause of CFP is still not finally determined, although CTX is the prime contender. However, MTX and MTX analogues may also have a role, and symptoms may be confused if Pltxs, prorocentrolides (produced by *Prorocentrum* species) or cooliatoxins (produced by *Coolia* species) are present.

The *Coolia malayensis* isolates in the CICCM (Table 1) have underpinned phylogenetic and toxicological studies into this new species (Rhodes *et al.* 2014c; Wakeman *et al.* 2014). The genus is widely distributed globally with some species known to be toxic.

The CICCM (Figure 2) will continue to provide cultures for on-going research and available cultures can be ordered via the CICCM website.

Acknowledgements

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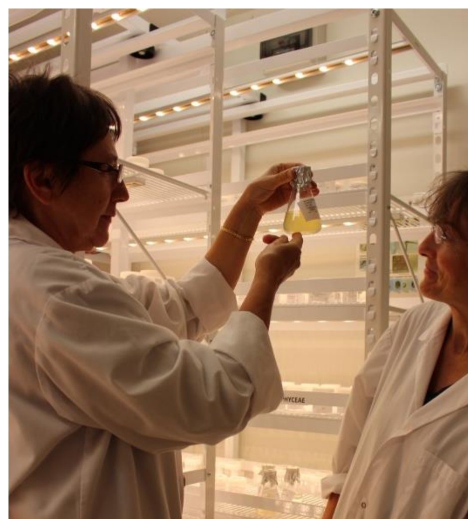

Fig. 2. The new EnviroTech Wing, Cawthron Institute, Nelson, New Zealand (top), which houses the Cawthron Institute Culture Collection of Micro-algae (CICCM). Curator Krystyna Ponikla (left lower image) and assistant curator Sarah Challenger (right) in the new fully alarmed, LED-lit and temperature controlled culture collection room.

HAB Transcriptomics

Detection of cyanotoxin genes in freshwater benthic cyanobacteria from sub-tropical south-eastern Australia using multiplex real time PCR

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Abstract

Benthic cyanobacterial mats collected from a range of freshwater lakes, rivers and creeks in the sub-tropics of Queensland (Australia) were tested for the presence of cyanotoxin genes by multiplex-tandem PCR. We were able to rapidly detect genes which encode anatoxin, cylindrospermopsins, microcystins/nodularin and paralytic shellfish toxins from a number of ecologically diverse sites. Sequencing of the partial *anaC* gene which encodes anatoxin indicated the likely source of the gene to be *Oscillatoria* and/or *Phormidium*, while the source of the *mcyE/ndaF* gene (encoding microcystins/nodularin) was *Nodularia*. We have found evidence that Australian freshwater benthic cyanobacteria have the genetic capacity to produce anatoxins which has not previously documented in Australia. There was also evidence of a genetic determinant essential to nodularin biosynthesis associated with a freshwater species putatively identified as *Nodularia moravica,* a species currently not known to be toxigenic.

Keywords: benthic, cyanobacteria, cyanotoxins, MT-PCR

Introduction

Cyanotoxins can represent a risk to human, animal, and ecosystem health when present in large quantities in either drinking or recreational waters. In Australia, cyanotoxin production by freshwater planktonic cyanobacteria has been well-studied and documented but there has been relatively little characterisation of freshwater benthic cyanobacteria, particularly species that form dense proliferations. In 2012 a pilot study of benthic cyanobacterial mats from lakes and streams in subtropical southeastern Australia showed that nearly 25% of samples contained cyanotoxin genes when screened by multiplex tandem real time PCR (MT-PCR). The aim of this project was to extend screening of benthic mats to other sites and potentially identify candidate toxin-producing cyanobacterial genera.

Material and Methods

Twenty eight benthic samples were collected from lakes, rivers and creeks in south-east Queensland (Australia). Dominant cyanobacterial genera were identified by phase contrast and Nomarski differential interference contrast microscopy with an Olympus BX51 (Tokyo, Japan) compound microscope. Photomicrographs were taken using an Olympus SC100 digital microscope camera.

The MoBio® PowerPlant Pro Kit (MoBio Labs, California, USA) was used to extract DNA from 15–90 mg (wet weight) sample. The presence of genes involved in the synthesis of toxins from four of the major cyanotoxin groups (cylindrospermopsins, microcystins, nodularin and paralytic shellfish toxins) was assessed by MT-PCR using the AusDiagnostics Cyanobacteria EasyPlex Kit and the Gene-Plex robotic platform as described in Baker *et al.* (2013). Samples were also screened by conventional PCR for the *anaC* gene found in anatoxin-producing cyanobacteria (Rantala-Ylinen *et al.* 2011).

PCR products from some sites positive for the *anaC* and *mcyE/ndaF* genes, encoding anatoxin (ATX) and microcystins/nodularin (MCY/NOD) respectively, were directly sequenced (Macrogen, Seoul, Korea). Consensus data was generated using ChromasPro134 software (Technelysium Pty Ltd, Brisbane, Australia).

Results and Discussion

The benthic cyanobacterial flora at many of the sites was dominated by members of the Oscillatoriales but Nostocalean species such as

Anabaena and *Nodularia* were also present (Fig. 1).

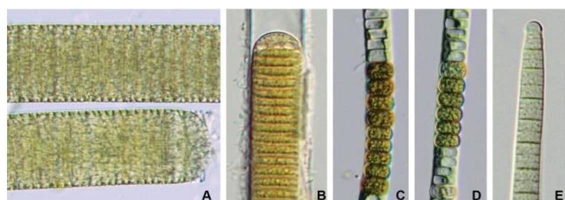

Fig. 1. Benthic cyanobacteria identified from mats. *Oscillatoria* spp. (A & B), *Nodularia moravica* (C & D), *Phormidium* sp. (E)

The MT-PCR analysis indicated that in total, 11 sites were positive for one or more of the five cyanotoxin genes tested. Four sites were positive for both the *anaC* and the *mcyE/ndaF* genes; two of these were also positive for the *cyrC* gene. The *sxtI* gene was identified at one site only.

Table 1. Dominant cyanobacteria and probable source of toxin gene as determined by sequencing and phylogenetic analysis

Site	Dominant species	Source of gene
ECY1	*Nodularia moravica* *Nostochopsis lobatus*	possibly *Nodularia* (*mcyE/ndaF*)
ECY2	*Oscillatoria sancta*	*Oscillatoria* sp. (*anaC*)
ECY4	*Oscillatoria sancta* *Anabaena inequalis* *Phormidium tergestinum*	*Oscillatoria* sp. (*anaC*); possibly *Nodularia* (*mcyE/ndaF*)
ECY10	*Merismopedia glauca* *Anabaena inequalis* *Nodularia moravica* *Phormidium* sp.	*Oscillatoria* sp. (*anaC*); possibly *Nodularia* (*mcyE/ndaF*)
ECY11	*Oscillatoria sancta* *Phormidium* sp.	*Oscillatoria* sp. (*anaC*); possibly *Nodularia* (*mcyE/ndaF*)

ATX-producing cyanobacteria have not been reported from Australia so further analysis was required to identify the potential sources of the *anaC* gene in the benthic mats. Sequencing (Table 1) and phylogenetic analysis (Fig. 2) of *anaC* amplicons showed that the gene was likley to have originated from *Oscillatoria* at sites ECY2, ECY4 and ECY11, which is consistent with the dominant species observed at each site. Although *Oscillatoria* was not dominant at site ECY10, another potential anatoxin producer, *Phormidium,* was instead present. Unfortunately, there is no sequence information available for the *anaC* gene in *Phormidium* for comparison with *Oscillatoria*.

Sequencing and phylogenetic analysis (not shown) of partial *mcyE/ndaF* genes generated by PCR indicated *Nodularia moravica* might be the origin of these homologous genes which are implicated in MCY/NOD biosynthesis. As *N. moravica* is a freshwater species in which nodularin production is suspected but not proven (Staykova *et al.* 2010), the presence of a novel, freshwater nodularin producer is an exciting finding as *N. spumigena* is the only known producer of this toxin in Australia (McGregor *et al.* 2012).

MT-PCR has proven to be a rapid and simple method of identifying cyanotoxin genes in benthic cyanobacterial mats from south-eastern Australia. We have isolated numerous single-trichome strains of candidate ATX and MCY/NOD producers from sites which were positive for cyanotoxin genes; these include *Calothrix, Geitlerinema, Oscillatoria, Phormidium* and *Scytonema.* These will be screened for cyanotoxin genes by PCR and further analysed for the production of toxins by LC-MS.

The *sxtI* and *cyrC* amplicons will also be sequenced to identify the potential origin of the genes, providing an indication of the cyanobacterial species that should be targeted for isolation and further molecular and chemical analysis.

In Australia, the distribution of potentially toxigenic benthic cyanobacteria in reservoirs and dams for drinking water is currently unknown. Eventually these water bodies will be screened for benthic cyanotoxin production so that an informed risk assessment of their potential impact on human health can be made.

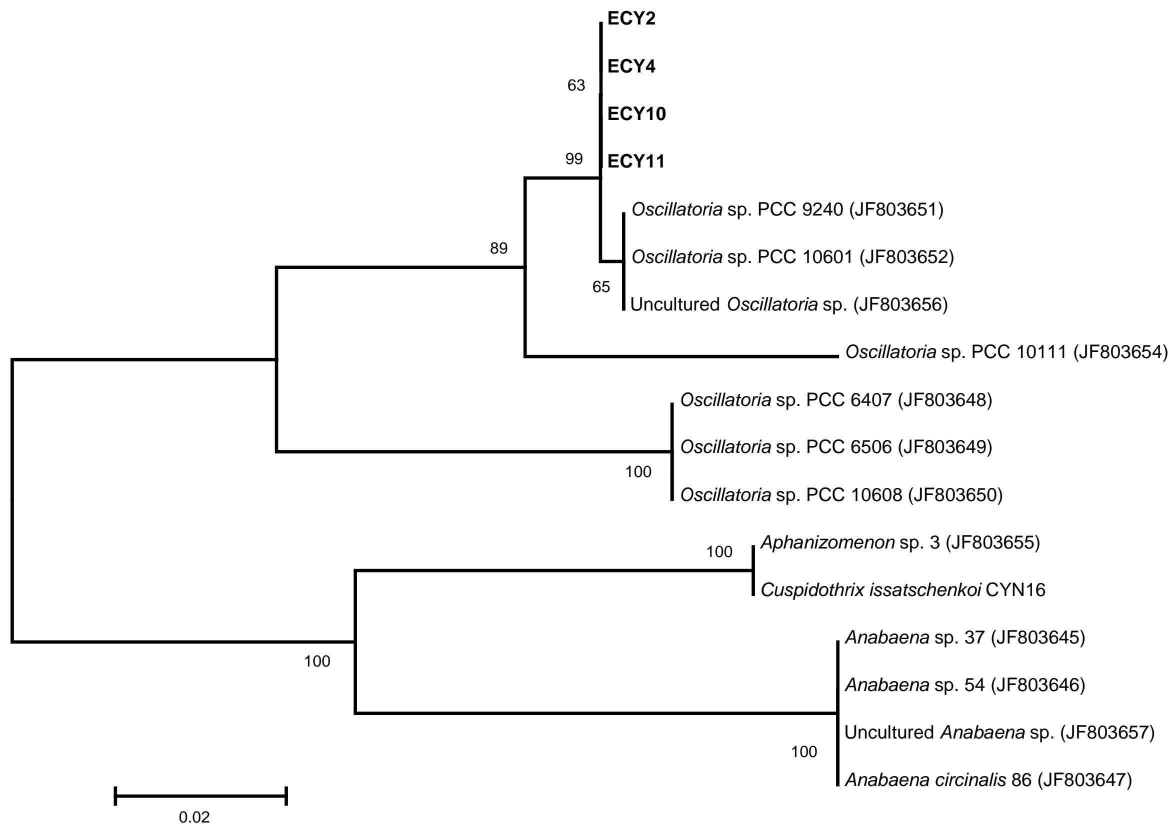

Fig. 2. Phylogenetic tree based on the *anaC* gene sequence (365 bp) generated by maximum likelihood (ML) analysis (1000 bootstrap replicates). Scale bar=no. nucleotide substitutions per site

Acknowledgements

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Comparative transcriptomic analysis of three toxin-producing *Karenia* species

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Abstract

Transcriptomic data for three *Karenia* species *(K. brevis, K. papilionacea, K. mikimotoi)* were compared to identify potential *Karenia* orthologs and investigate putative peptides involved in brevetoxin biosynthesis. Recent results have shown that *K. papilionacea*, like *K. brevis*, produces brevetoxin (PbTx-2). In contrast, *K. mikimotoi* does not make brevetoxin but instead produces gymnocin, another type of ladder-frame polyether. Reference transcriptomes for each species were assembled using high-throughput sequencing technology and the *de novo* assemblers Velvet-Oases and Trinity. Orthologous putative proteins were identified among *Karenia* transcriptomes using the reciprocal BLAST method and annotated with the NCBI non-redundant protein database and InterProScan. We identified twenty-one type 1-like putative polyketide synthases and one putative epoxide hydrolase-like peptide that were expressed in *K. brevis* and *K. papilionacea*, but not *K. mikimotoi*. These enzymes represent potential steps in the brevetoxin synthesis pathway. Additionally, a database of 3,495 ”apparently unique” *K. brevis* and *K. papilionacea* orthologous genes was created by querying the transcriptomes of twenty phytoplankton species. The unique orthologs provide valuable insight into the biology of brevetoxin-producing dinoflagellates.

Keywords: transcriptomics, harmful algae species, toxin production, dinoflagellates

Introduction

Karenia brevis, a bloom-forming dinoflagellate, is among the most prominent harmful algae species in the Gulf of Mexico. *K. brevis* cells produce ladder-frame polyether polyketide compounds called brevetoxin (PbTx) (Lin and Risk 1981; Shimizu et al. 1986). PbTx-1, PbTx-2, and their derivatives bind to neurotoxin receptor site 5 in mammalian voltage-gated sodium channels, thereby inhibiting channel deactivation (Huang et al. 1984; Baden 1989; Dechraoui et al. 1999). *K. brevis* blooms have caused neurotoxic shellfish poisoning incidents, fish kills, and marine animal deaths along the Gulf coast (Landsberg 2002). Despite the human health, environmental, and economic risks associated with brevetoxin, their biological function in *K. brevis* is poorly characterized, and the genes associated with brevetoxin production are currently unknown. It is hypothesized that standard polyketide synthase (PKS) acyl transferase (AT), ketosynthetase (KS), β-keto-reductase (KR), dehydratase (DH), and acyl carrier protein (ACP) catalytic domains participate in brevetoxin synthesis (Shimizu 2003). An limonene epoxide hydrolase-like enzyme may participate in polyether ring formation, much like the monesin model (Gallimore et al. 2006; Gallimore 2009). However, the brevetoxin biosynthetic mechanism is still under debate, particularly since recent radiolabelling work suggests that an oxidative reaction produces PbTx-1 and PbTx-2 from alcohol intermediates (Calabro et al. 2014).

PbTx-2 has been measured in *Karenia papilionacea* (Fowler et al. unpublished). To investigate potential genes underlying brevetoxin production, we assembled, compared, and functionally annotated the reference transcriptomes of *K. brevis*, *K. papilionacea*, and *Karenia mikimotoi* clones using high-throughput sequencing technology. A close phylogenetic relative to *K. brevis* and *K. papilionacea* (Haywood *et al*. 2004), *K. mikimotoi* does not produce brevetoxin, and was therefore an ideal control species during this experiment. By identifying apparently unique orthologs expressed by *K. brevis* and *K. papilionacea*, we aimed to elucidate the unique biology of brevetoxin-producing dinoflagellates. In particular, we searched for unique PKS and epoxide hydrolase sequences, because of their potential role in PbTx-2 biosynthesis.

Material and Methods

K. brevis Wilson, *K. papilionacea* CAWD91, and*K. mikimotoi* C22 cultures were maintained in

A. Lincoln MacKenzie [Ed]. *Marine and Freshwater Harmful Algae 2014*. Proceedings of the 16th International Conference on Harmful. Algae. Cawthron Institute, Nelson, New Zealand and the International Society for the Study of Harmful Algae (ISSHA)

L1 medium (Guillard and Hargraves 1993) at salinity 35 (*K. brevis* and *K. papilionacea*) or salinity 30 (*K. mikimotoi*). Triplicate 150-mL cultures of each species were cultured on a 12:12 hour light:dark cycle at 25 °C (*K. brevis*) or 20 °C (*K. papilionacea* and *K. mikimotoi*).

Table 1 MMETSP CAMERA data used during this project. The MMETSP sample IDs of each transcriptome are listed in parentheses.

Species	Species
Alexandrium fundyense CCMP1719 (0196, 0347)	*Isochrysis galbana CCMP1323* (0944, 0943, 0595)
Amphidinium carterae CCMP1314 (0258, 0398, 0259)	*Karlodinium micrum CCMP2283* (1016, 1015, 1017)
Aureococcus anophagefferens CCMP1850 (0916, 0914, 0917, 0915)	*Lingulodinium polyedra CCMP1738* (1034, 1032, 1035, 1033)
Ceratium fusus PA161109 (1075, 1074)	*Oxyrrhis marina LB1974* (1426, 1424, 1425)
Chaetoceros neogracile CCMP1317 (0754, 0752, 0751, 0753)	*Perkinsus marinus ATCC50439* (0922)
Crypthecodinium cohnii Seligo (0325, 0326, 0324, 0323)	*Prorocentrum minimum CCMP1329* (0053, 0055, 0057, 0056)
Ditylum brightwellii GSO104 (1010, 1013, 1012)	*Pseudo-nitzschia australis 10249_10_AB* (0139, 0140, 0141, 0142)
Dunaliella tertiolecta CCMP1320 (1126, 1128, 1127)	*Scrippsiella hangoei SHTV5* (0361, 0359, 0360)
Emiliania huxleyi CCMP370 (1155, 1154, 1156, 1157)	*Symbiodinium kawagutii CCMP2468* (0132)
Fragilariopsis kerguelensis L2_C3 (0906, 0909, 0907, 0908)	*Thalassiosira oceanica CCMP1005* (0971, 0972, 0970, 0973)

During the late exponential growth phase, cells in each bottle were pelleted via centrifugation. RNA was extracted from the pellets with the Qiagen RNEasy Mini Kit (Qiagen Inc., Valencia, CA) in accordance with kit protocol and stored at -80 °C until sequencing. The sample with the highest RNA concentration, as determined by NanoDrop Spectrophotometer, was sent on dry ice overnight to the Michigan State University Research Technology Support Facility (RTSF). RTSF prepared sequencing libraries with the Illumina Stranded mRNA Library Prep Kit LT, and 150 bp short reads were sequenced with the llumina HiSeq 2500 Rapid Run flow cell (v1). Base calling was performed using Illumina Real Time Analysis software v 1.17.21.3.

Short reads were trimmed for quality and length with CLC Genomics Workbench v 6.5 (CLC Bio, Aarhus, Denmark). A Phred quality threshold of 0.05 and minimum length threshold of 100 bp were enforced. Trimmed short reads were processed by the Trinity (default parameters) (Simpson et al. 2009) and Velvet-Oases (k-mer length 45) (Schulz et al. 2012) *de novo* transcriptome assemblers. Trinity and Velvet-Oases assemblies, including all predicted isoforms, were combined into complete reference transcriptomes for *K. brevis*, *K. mikimotoi*, and *K. papilionacea*. The longest potential open reading frame (ORF) in each transcript was extracted and converted to amino acids with longorf.pl (Kortschak 2002), thus producing peptide databases. Redundant peptides (≥ 99% similar) were trimmed with CD-HIT v 4.5.4 (Fu et al. 2002).

To identify orthologs among *Karenia* species, the peptide databases were compared with reciprocal BLASTp (Altschul et al. 1997), using a maximum E-value of 1.00E-20, according to protocol provided by the Harvard FAS Center for Systems Biology. Orthologs unique to *K. brevis* and *K. papilionacea* were annotated with a BLASTp search against the NCBI non-redundant database (maximum E-value of 1.00E-20) and the complete application suite included in InterProScan 5 (Mitchell et al. 2015). Orthologs in *K. brevis* and *K. papilionacea* were further compared to 20 phytoplankton reference transcriptomes from the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) (Table 1) using reciprocal BLAST, as above.

Results and Discussion

After removing redundancies with CD-HIT, the *K. brevis*, *K. mikimotoi*, and *K. papilionacea* peptide databases contained 147,200, 179,645, and 180,963 sequences, respectively. Approximately 80% of the putative peptides were >100 aa long (Fig. 1A), from continuous ORFs >300 bases. Short ORFs are more likely to occur randomly, to be incorrectly annotated during a protein BLAST search, or yield no statistically significant annotation results (Linial 2003). But predicted ORFs were not removed from the datasets based on length, to best support the goal of novel protein identification. After CD-HIT concatenation, more than 70% of the nonredundant peptide sequences

were from the Oases assembler, thus suggesting that Velvet-Oases created more complete ORFs than Trinity.

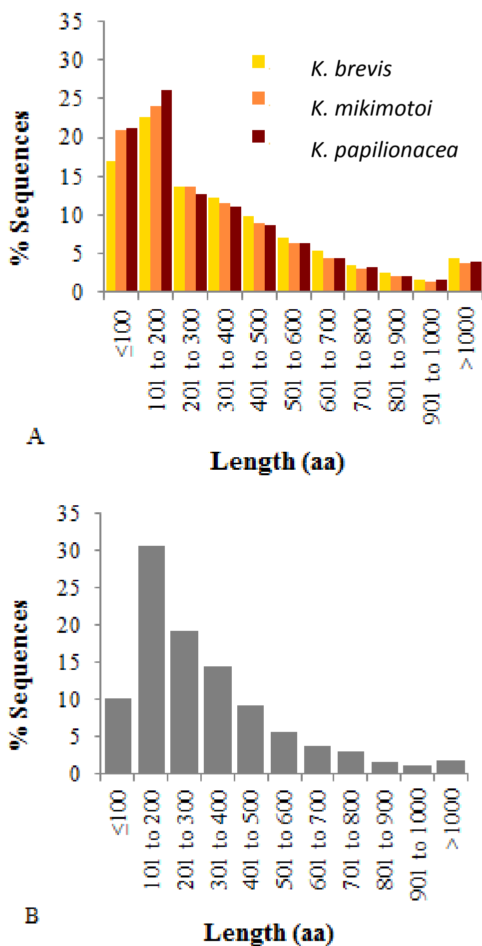

Fig. 1. Length distribution of (A) the complete *K. brevis, K. mikimotoi,* and *K. papilionacea* putative peptide databases and (B) the 3,495 apparently unique *K. brevis* and *K. papilionacea* peptides. Lengths in (B) are graphed based on the *K. brevis* ortholog data.

The reciprocal BLASTp search identified 70,032 sequences in the *K. brevis* transcriptome with potential orthologs expressed by *K. mikimotoi* and/or *K. papilionacea* (Fig. 2). Of these, 6,561 orthologs were expressed by *K. brevis* and *K. papilionacea*, but not *K. mikimotoi*. After querying the twenty MMETSP transcriptomes, the pool of "apparently unique" *K. brevis* and *K. papilionacea* orthologs decreased to 3,495. Over 90% of the "apparently unique" peptide sequences were > 100 aa long (Fig. 1B). Only 8.24% of the "apparently unique" peptides significantly matched one or more nr sequences with an E value ≤ 1.0E-20. This low annotation rate is expected from a database of *K. brevis* proteins with no orthologs in close phylogenetic relative *K. mikimotoi* or any of the MMETSP representative species. In contrast, InterProScan successfully assigned a protein family, repeat, domain, and/or site to 64.12% of the apparently unique putative proteins, suggesting that 1,953 unique sequences with no nr annotation may nevertheless contain short conserved protein motifs.

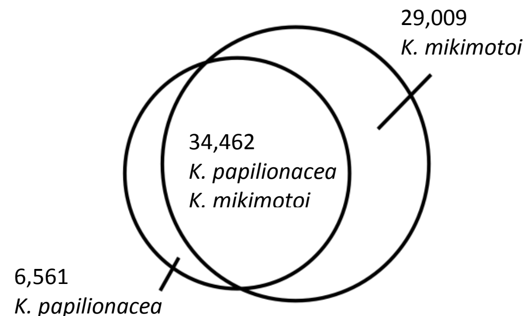

Fig. 2. Number of peptides from the *K. brevis* reference transcriptome with probable orthologs in one, both, or neither of the *K. mikimotoi* and *K. papilionacea* transcriptomes. Orthology was predicted with a reciprocal

K. brevis expressed 21 putative PKSs in common with *K. papilionacea,* but not *K. mikimotoi,* orthologs. Of these, four had a predicted AT domain, one had a predicted KS domain, three had a predicted KR domain, one had a predicted DH domain, 10 had a predicted ER domain, and five had a predicted ACP domain. Nineteen of the PKSs had only one catalytic domain.

Only the KS-containing ortholog was an "apparently unique" sequence, since the other PKSs had at least one orthologous match in the group of MMETSP phytoplankton transcriptomes. The KS domain catalyzes carbon bond formation (Claisen condensation) in polyketide skeletons and is highly conserved among eukaryotes (Keatinge-Clay 2012). We identified the KS cysteine (TACSSS) and histidine (EAHGTG and KSNIGHT) motifs (Keatinge-Clay 2012) in the apparently unique *K. brevis* and *K. papilionacea* PKS (Fig. 3). One "apparently unique" epoxide hydrolase-like sequence was expressed by *K. brevis* and *K. papilionacea*. With significant BLAST similarity to bacterial limonene epoxide hydrolases in the nr database, it was 394 aa long and 76.9% identical between the two *Karenia* species. InterProScan identified a conserved N-terminus epoxide hydrolase protein motif (pfam ID 06441) from amino acid 19 to 131.

K. brevis	FHCDTACSSTNVT
K. papilionacea	QHIDTACSSSNVA
cysteine	----TACSSS---
K. brevis	TTCELHGTGTALG
K. papilionacea	TTTELHGTGTALG

Fig. 3. Cysteine and histidine catalytic regions in the *K. brevis* and *K. papilionacea* "apparently unique" KS domain-containing PKS. *Karenia* sequences are aligned to each other and the highly conserved concensus motif.

Based on comparative transcriptomics, the *K. brevis* total transcriptome contains more sequences that are orthologous to genes in the *K. mikimotoi* transcriptome than the *K. papilionacea* transcriptome (Fig. 2). This result is not unexpected, since *K. mikimotoi* is the closer phylogenetic relative to *K. brevis*, based on rDNA sequences (Haywood et al. 2004). Nevertheless, this study identified >3000 predicted orthologs coding putative peptides >100 aa long that were "apparently unique" to *K. brevis* and *K. papilionacea*, based on reciprocal BLAST searches against 21 phytoplankton species, including *K. mikimotoi*. Of particular interest are the 21 PKS sequences that were expressed by *K. brevis* and *K. papilionacea*, but not *K. mikimotoi*, including the KS domain-containing peptide without an identified ortholog among the MMETSP transcriptomes. Most of the PKS transcripts had a single catalytic domain, similar to eight type 1-like PKS sequences that have been identified previously in *K. brevis* (Monroe and Van Dolah 2008). The apparently unique putative limonene epoxide hydrolase-like peptide is another intriguing target for future research as a step in brevetoxin synthesis. The novel genes identified in this comparative transcriptomic study of brevetoxin-producing dinoflagellates has produced a wealth of genes for further study.

Acknowledgements

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Discovery of non-coding RNAs in *Amphidinium carterae* differentially expressed over a diel cycle

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Abstract

Although some proteins in dinoflagellates are differentially expressed over a diel cycle, mRNA transcripts for these genes appear not to change in concert. This implies that protein expression is controlled post-transcriptionally. We extracted RNA from *Amphidinium carterae* (CCMP1314) at thirteen time points across the light/dark transition. Using RT-qPCR, expression of genes for metabolism, photosynthesis, translation initiation, as well as ribosomal RNAs showed little differences. Size selected small RNA libraries (<200 bases) were constructed for each time point, and Illumina sequencing was performed resulting in 2770 unique assemblies. Expected rRNAs, tRNAs, snRNAs, an 80 base putative spliced leader RNA, and organelle mini-circle gene fragments were recovered; but an unexpected abundance of additional ncRNAs were also recovered. Of the 2770 sequences produced, 1454 were expressed only at a single time point. The remaining 1316 clustered into 17 discrete expression profiles, of which three were similar to the RT-qPCR diel pattern and 14 represented novel expression profiles. Although several traditional miRNAs were annotated, most of the ncRNAs could not be annotated and may be part of a yet undescribed pathway.

Keywords: small dinoflagellate ncRNA diel cycle expression

Introduction

Dinoflagellates possess unique molecular traits that differ from 'classical' model organisms. Along with the idiosyncrasies of the dinokaryon (Kato *et al*. 1997), characteristic protein changes associated with circadian cycles cannot be correlated with changes in messenger RNA (mRNA) transcript abundance (Mittag and Li and Hastings 1998, Van Dolah *et al*. 2007), indicating a post-transcriptional mechanism of gene regulation. This makes the measure of transcript abundance inadequate to assess dinoflagellate responses to environmental stimuli or to answer questions about their basic biology.

Non-coding RNAs (ncRNAs) are involved in many eukaryotic cellular processes, both regulatory and catalytic. The most obvious example is ribosomal RNAs (rRNAs), which perform both structural and catalytic roles. There are also the eukaryote specific intron splicing RNAs, U1 through U6, and the spliced leader RNA (Reddy *et al*. 1983, Lidie and Van Dolah 2007, Zhang *et al*. 2009).

MicroRNAs (miRNAs) are an example of a non-coding RNA that can regulate gene expression post-transcriptionally. They are characterized by their method of synthesis and affect gene expression by modifying chromosome structure, inhibiting transcription, initiating degradation of mRNAs, or inhibiting translation (Kim 2005; Djuranovic, Nahvi, and Green 2011). Eight small RNAs (smRNAs) and 13 miRNAs were recently described in *Symbiodinium microadriaticum* across multiple conditions and could be assigned to known genes (Baumgarten *et al*. 2013), demonstrating the existence of this specific type of ncRNA as well as the core RNA interference (RNAi) protein machinery in dinoflagllates. However, these ncRNA libraries were constructed using adaptors specific for RNAs generated by the enzyme Dicer and showed limited differential expression. This is not surprising since miRNAs and smRNAs are associated with cellular differentiation and, although this method of post-transcriptional regulation is important in other organisms, it is unlikely to regulate basic circadian and metabolic processes in dinoflagellates. In order to test the hypothesis that dinoflagellate ncRNAs can be differentially expressed, size fractionated RNA from *Amphidinium carterae* (CCMP1314) was isolated and quantified during a diel cycle and compared to levels of mRNA and rRNA.

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Material and Methods

Amphidinium carterae (NCMA #1314) was grown in ESAW medium (Berges *et al*. 2001) with 1 mM HEPES in a 20 L polycarbonate carboy. The culture was grown on a 14:10 light:dark schedule at 150 µmole m^{-2} s^{-1} with bubbling air infused with CO_2. Culture pH was regulated with a pH controller (pH 7-6 to 8.2). Two liters of fresh medium was exchanged Monday, Wednesday and Friday to a cell density of 172,000 cells/ml. Aliquots of 250 ml were dispensed into twenty six 75 cm^2 polystyrene culture flasks placed to maintain identical light exposure in two rows of thirteen with duplicates in an opposing direction. Each duplicate pair was harvested according to the following schedule relative to lights off in hours: -6.0, -4.0, -2.0, -1.5, -1.0, -0.5, 0, 0.5, 1.0, 1.5, 2.0, 4.0, and 6.0. 50 ml samples were centrifuged at 1000 x g for 10 min at 20° C and suspended in 1 ml of tri reagent (Sigma-Aldrich) and extracted according to the manufacturer's protocol. RNA was quantified on a Nanodrop 1000 (Thermo Fisher by Life Technologies) and also on a Qubit 2.0 fluorometer (Life Technologies). Quality and relative sizes of prominent bands were assessed on an Agilent 2100 Bioanalyzer using the RNA 6000 and small RNA kits according to the manufacturer's protocols. RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen by Life Technologies) with Random Primers (Invitrogen) according to the manufacturer's protocol. Quantitative real-time PCR (qPCR) was performed using an Applied Biosystems Fast 7500 thermal cycler in duplicate with the following reaction setup: 6 µl of nuclease free water, 2 µl of forward and reverse primers at 5 µm each, 10 µl of iTaq 2X master mix containing SYBR green and ROX (Bio-Rad), and 2 µl of template cDNA at 10 ng/µl. Thermal cycling conditions consisted of an initial denaturation at 95° C for 2 minutes followed by 40 cycles of denaturation at 95° C for 15 seconds, annealing and fluorescent data collection at 60° C for 30 seconds, and extension at 72° C for 30 seconds. Melt curves were performed to ensure product quality. Cycle thresholds and baselines were determined manually and relative quantities were determined using a 0 input CT of 38 and assuming a primer efficiency of 1.85 copies/cycle.

Total RNA from one replicate of each time point was size selected for fragments between 200 and 30 bases using a BluePippin electrophoresis system (Sage Science) on a 2% TBE cassette. Isolated RNA was ligated with unique indices and Illumina adaptors, and amplified to generate libraries according to the Truseq sample preparation guide V2. The libraries were sequenced using an Illumina MiSeq. The resultant 190 base un-paired reads following adaptor removal were trimmed to the first base with a quality score below 60. Reads from all time points were assembled *de novo* using CLC genomics workbench 7.1 (Qiagen). The PhiX standard was removed and sequences were re-assembled using Sequencher 5.2.3 (Gene Codes Ann Arbor, MI) to combine duplicates. Sequences were annotated using miRBase, BLAST and tRNAscan (Schattner *et al*. 2005). The reads were mapped back to the master contig list, and expression levels were calculated as reads per thousand bases per million reads (RPKM) for each time point. Contigs with expression at one time point greater than the sum of all other time points were classified as singularly expressed. For the remaining contigs,

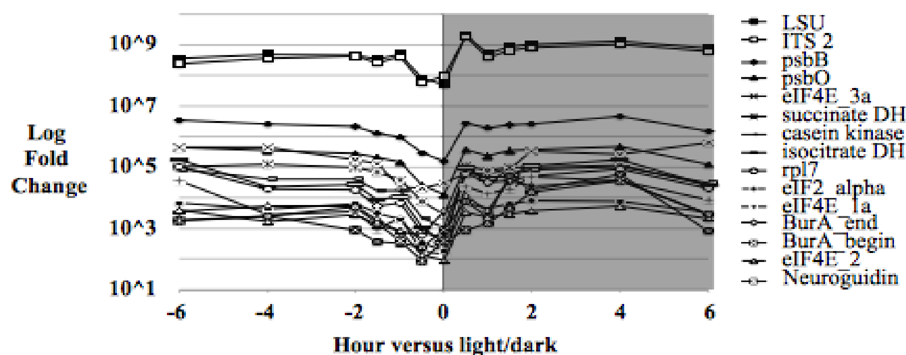

Fig. 1. Estimated expression of 13 mRNAs as well as the large subunit ribosomal and the internal transcribed spacer 2.

RPKM was normalized by conversion to a fold change relative to the -4.0 timepoint. The normalized data were then compared using Cluster 3.0 with Spearman Rank correlation and

divided into 17 clusters using Treeview 1.16r4 (Saldanha. 2004). Mean and median time point values were calculated for each cluster.

Results and Discussion

The qPCR data for 14 RNAs show a steady-state followed by a drop in abundance prior to lights off and then a return to steady state (Fig. 1). This was true for ribosomal targets as well as genes involved in photosynthesis (psbO, psbB), general metabolism (isocitrate, succinate dehydrogenase), and gene regulation (casein kinase), among others. We presume this is due to an interruption of transcription just prior to cell division.

The similarities in profiles between genes expected to have differential expression of proteins over the diel cycle and that of the ribosomal gene corroborates a lack of transcriptional regulation and indicates global changes in the transcriptome over the cell cycle.

Of the 2770 resultant sequences obtained from the fraction of RNA less than 200 bases, 1454 were determined to have expression at a single time point with little or no expression at all others, including 39 of the 42 putative microRNAs identified by miRBase. These were binned by RPKM, and a histogram of these results is shown in Figure 2.

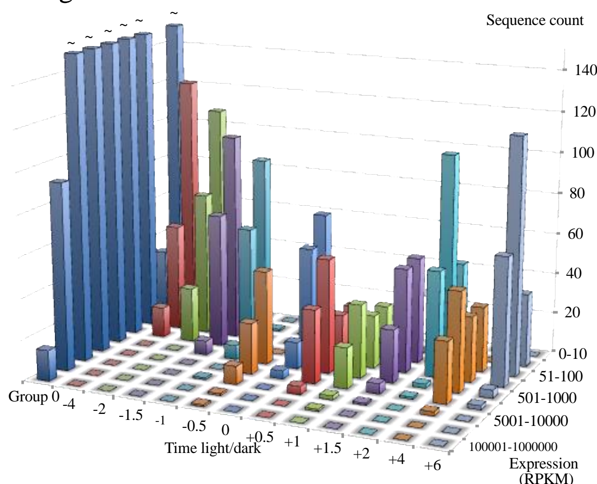

Fig. 2. Histogram of the RPKM values for sequences expressed at a single time point.

The read counts were often low at each time point whereby the sequence length was usually the main determinant for RPKM and ranged from 17 to 261 bases with a median of 75. However, singular expression occurred at all time points with similar frequencies; including times -0.5 and 0.0 when mRNAs were at a relative low abundance. Also, none of the reads generated by this study mapped to genomic sequences for the extremely abundant transcripts actin, major basic nuclear protein, and psbO; including annotated introns. This makes it unlikely that these ncRNAs are introns, gene fragments, or breakdown products of mRNAs during turnover. The remaining 1316 sequences clustered into 17 expression profiles. This included sequences identified by BLAST as nuclear and organellar ribosomal genes, fragments of organellar ORFs, and transfer RNAs (tRNAs) (Fig. 3). Although the ribosomal genes and organellar ORFs did not cluster together, they were placed into clusters all showing the characteristic reduction prior to the light/dark transition and a subsequent recovery, *e.g.* profiles I, II and VI. Profile I in particular is analogous to a plot of the reads generated for each time point and the most similar to the qPCR results. This was not true for the tRNAs, which were placed in clusters with a variety of expression profiles, including clusters VII and VIII, which both show an increase in relative abundance prior to the light/dark transition, in opposition to the qPCR results. tRNA abundance may be a way of kinetically controlling gene expression for proteins with an abundance of certain amino acids and are one possible mechanism of post-transcriptional regulation.

The role of the unannotated ncRNAs and how differential expression is accomplished is still unclear. Most of the ncRNAs are polyadenylated, which, along with polyuridination, has been shown to affect RNA half-lives (Zhuang and Zhang and Lin 2013). Although these mechanisms certainly play a role in observed abundance they cannot fully explain the diverse range of expression profiles observed in this study. The putative miRNAs are likely similar to those in other eukaryotes in synthesis and function, but the majority of the ncRNAs are too long and lack structures common to hairpin forming RNAs.

Thus, the unannotated ncRNAs are unlikely to be novel miRNAs. The most abundant ncRNAs are present in the tens or hundreds of thousands of reads, and were expressed constitutively. This

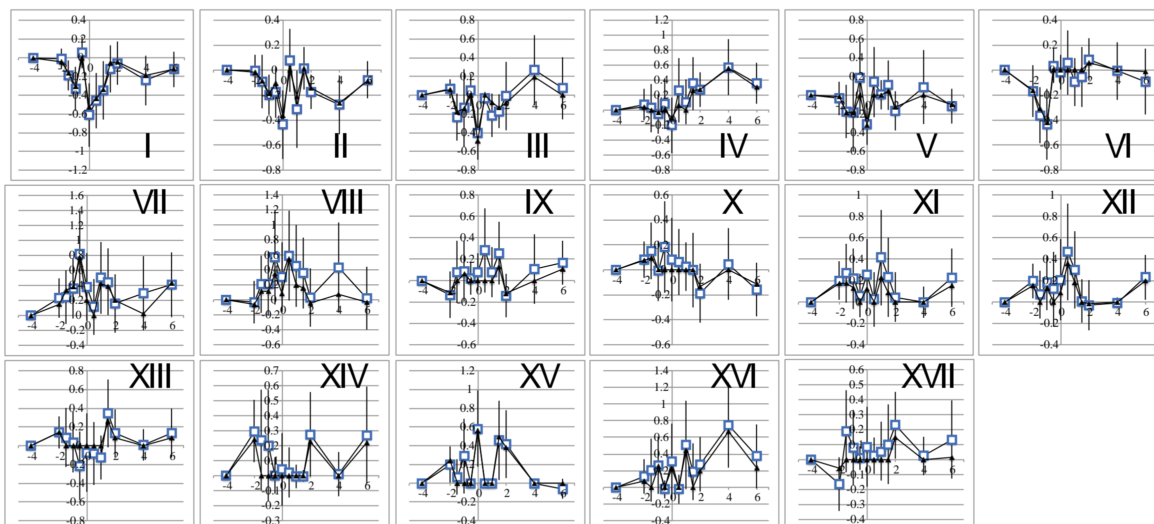

Fig. 3. Expression profiles generated by cluster analysis. Average (blue squares) and median (black triangles) values are shown. Standard deviations are given to highlight times within each cluster with high variability.

makes it unlikely that most of the ncRNAs act by degrading or inhibiting mRNAs. Although some of these RNAs may structurally interact with DNA, it is unlikely that the bulk of the ncRNAs act as chromosome remodelers since large-scale interactions with chromatin are more likely to result in transcriptional effects, which are noticeably absent in dinoflagellates.

One possibility is that the unannotated ncRNAs are linked to the initiation of a cellular process. These could be quite varied given the diverse range of expressions described in this study, and could include regulation of gene expression. Also, members of profiles VII and VIII may be involved in replication as Okazaki primers or guides for local chromosome decondensation. However, it is unclear if we are observing these sequences following synthesis, possibly through direct transcriptional control or an enzymatic process; or if they are the end product of another reaction such as a splicing event or cleavage of a structural RNA. In summary, this is the first description of RNAs differentially expressed in a dinoflagellate. Further investigations into the interacting partners of these ncRNAs may elucidate both their functions and the mechanisms that have created them.

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Translating the message: *Karlodinium veneficum* possesses an expanded toolkit of protein translation initiation factors

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Abstract

Dinoflagellates are unusual eukaryotes with large genomes and a reduced role for transcriptional regulation compared to other eukaryotes. The mRNA in dinoflagellates is *trans*-spliced with a 5′-spliced-leader sequence, yielding a 22-nucleotide 5′-sequence with a methylated nucleotide cap. Since the control of gene expression is primarily post-transcriptional, this study focuses on mRNA recruitment as a means for regulating gene expression and specifically on the diversity of eIF4E family members. Three novel clades related to the cap binding initiation factor eIF4E have been recognized in alveolates that are distinct from the three metazoan classes of eIF4E. We have analyzed the characteristics of five of the fourteen eIF4E family members from *Karlodinium veneficum*, four from clade 1 and one from clade 2. Members of each clade all bear the distinctive features of a cap-binding protein. We examined their ability to interact with the cap analogue, m^7GTP using an *in vitro* bead-binding assay. We show that recombinant eIF4E-1 family members are able to bind the cap analogue m^7GTP, but eIF4E-2b binds poorly. Overall, the eIF4E-1 family members may be serving as general cap-binding translation initiation factors, while the eIF4E-2 (and perhaps eIF4E-3) family members may serve a regulatory role in gene expression.

Keywords: dinoflagellate, mRNA recruitment, eIF4E diversity

Introduction

Karlodinium veneficum is a small (<20 μm), non-thecate, mixotrophic dinoflagellate that causes toxic blooms worldwide. As a dinoflagellate, its genome is moderately large, about 5 times the size of the human genome (LaJeunesse *et al.*, 2005). In addition, as is true of dinoflagellates as a whole, genes are transcribed and then capped by *trans*-splicing yielding a unique spliced leader sequence (Lidie and Van Dolah, 2007; Zhang *et al.*, 2007) that may contain a complex cap structure. The size of their genomes is in part due to an extraordinary level of gene duplication that is exemplified by a large diversity of cap-binding translation initiation factor eIF4E-related proteins (Jones *et al.*, 2015).

In model eukaryotic organisms, *e.g.* mice, yeast, and fruit flies, gene regulation is a process regulated by transcriptional, translational, and post-translational mechanisms. However, dinoflagellates exhibit little transcriptional regulation of gene expression (Morey and Van Dolah, 2013; Roy and Morse, 2013), but do show changes in the proteome (Lee *et al.*, 2009; Liu *et al.*, 2012). Dinoflagellates appear to have independently evolved a complex system for regulating gene expression that may require a large toolkit of translation factors.

Due to the limited transcriptional control of gene expression exhibited by dinoflagellates and the presence of mRNA that is not always utilized, the question arises of how individual mRNAs are selected to be translated? The diversity of the eIF4E family may provide a selection mechanism for differential recruitment of specific mRNAs to the translational machinery (Hernandez *et al.* 2005; Joshi *et al.*, 2005). Each eIF4E may interact with mRNA to sequester it from, or specifically recruit it for, translation.

A previous phylogenetic study of the eIF4E family using transcriptome data revealed three clades of eIF4E with varying degrees of conservation among the family members. Based on amino acid alignments, some are predicted to serve as functional cap binding initiation factors while others are anticipated to play more regulatory roles in protein expression (Jones *et al.*, 2015). The three clades of dinoflagellate eIF4E family members, with bootstrap support >70 %

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further segregate into 9 subclades (Figure 1). Of all the family members, eIF4E-1a is the most duplicated with three distinct, yet conserved (>83 % identity), members. From these 14, we selected five *K. veneficum* eIF4E family members, eIF4E-1a1, -1a2, -1d1, -1d2, and eIF4E-2a. The selection was based in part on having Sanger sequencing support for each, for comparison of clade 1 eIF4Es with differences at the site for interaction with other proteins and for comparison of functional differences of clade 1 versus clade 2 eIF4E family members. We conducted a preliminary biochemical analysis of these five eIF4E family members from the dinoflagellate *K. veneficum*.

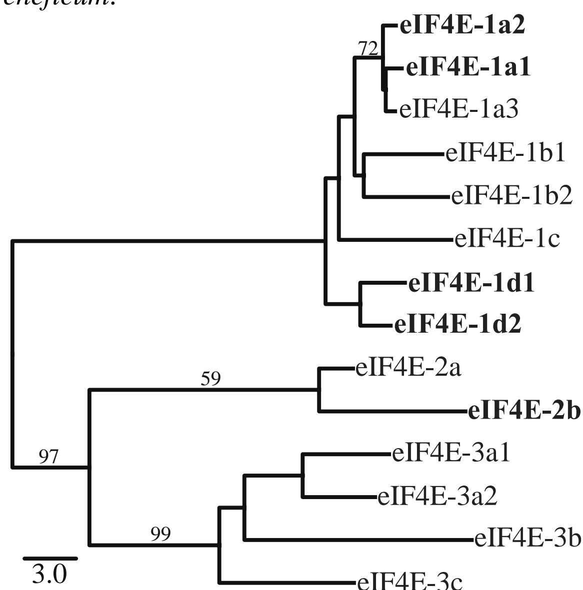

Fig. 1. This tree shows the *Karlodinium veneficum* eIF4E family members and their relationship to each other (Jones *et al.* 2015). *K. veneficum* has a greater degree of duplication of certain family members, *i.e.* eIF4E-1a and eIF4E-1b, than other dinoflagellates. The eIF4E members that were used in this study are highlighted with bold lettering.

Material and Methods

Synthesis and cloning of eIF4E constructs into an *in vitro* expression vector:

Nucleotide sequences for *K. veneficum* eIF4E family members, eIF4E-1a1, -1a2, -1d1, -1d2, and a single eIF4E-2 family member, eIF4E-2b, were codon optimized for rabbit, *Oryctolagus cuniculus*, using Advanced OptimumGene™ (Genscript). The nucleotide sequence was synthesized by Genscript and cloned into the *in vitro* transcription/translation plasmid vector pCITE-4a (+) (Novagen), using the NcoI and BamHI sites, which adds an S-tag to the amino-terminus and includes a stop codon at the carboxy terminus.

In vitro transcription/translation of eIF4E and luciferase:

In vitro translation (IVT) of each eIF4E was performed using the TNT Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's protocol. In brief, approximately 1 µg of plasmid DNA and 20 µCi of Easy-tag ^{35}S-methionine (Perkin Elmer) were mixed with a final volume of 50 µl of TNT to give a final concentration of 80 % TNT. Transcription and translation were carried out at 30 °C for 1.5 h. 1 µl of the IVT reaction was taken for analysis of ^{35}S-methionine incorporation by mixing to a final concentration of 5 % TCA, boiling and capturing on GF/C filter paper. Fractions were counted in Ecoscint Original scintillation cocktail (National Diagnostics) and cpm was determined using a LS6500 Multipurpose Scintillation Counter (Beckman Coulter).

7-methyl-guanosine-triphosphate binding assay:

Sepharose beads bound to 7-methyl-guanosine-triphosphate (Jena) were blocked using 1 mg/ml soy bean trypsin inhibitor (Sigma, T9128) in binding buffer (25 mM HEPES/KOH pH 7.2, 10 % glycerol, 150 mM KCl, 1 mM dithiothreitol, 1 mM D-L methionine) for 1 h at 4 °C with 1400 rpm shaking in a benchtop thermomixer 22331 (Eppendorf). The beads were washed twice with binding buffer without soybean trypsin inhibitor and suspended in 50 % v/v binding buffer. 20 µl of each IVT reaction was diluted 10-fold with binding buffer containing 200 µM GTP and 200 µM $MgCl_2$ and mixed with the bead suspension. Binding conditions were at 4 °C for 1 h shaking at 1400 rpm. The supernatant containing the unbound fraction was recovered by centrifugation at 500 x g at 4 °C. An equivalent of 1 µl of the original IVT was used for TCA precipitation and filtered onto a GF/C membrane (Millipore). The cap-analogue beads were washed five times with binding buffer and the final bead fraction was suspended in SDS-PAGE sample buffer. The bead suspensions were heated to 90 °C and a fraction equivalent to 1 µl of the original IVT reaction was applied to GF/C filter paper and counted as described above. IVT, unbound, and bead bound fractions were diluted in SDS-PAGE sample

buffer and heated to 90 °C for 3 min. The samples were separated by 17.5 % high-Tris SDS-PAGE using PAGEruler pre-stained molecular weight ladder (Fermentas) as a guide and transferred to Immun-blot PVDF (BioRad) using a Criterion blotter (BioRad) for 30 min at 100 V in 20 % methanol Towbin buffer (BioRad). Labeled proteins were visualized on the PVDF membrane using a Storage Phosphor screen (Molecular Dynamics) and imaged with a Typhoon 9410 Variable Mode Imager (GE Healthcare).

Results and Discussion

There are fourteen eIF4E family members present in the transcriptome of *Karlodinium veneficum:* Each eIF4E was expressed at relatively similar levels according to their FPKM, which ranges from 2.6 to 11.1 (Table 1). The amino acid length for each eIF4E ranged from 212 to 901 residues, with molecular weights ranging from 23.69 to 99.4 kDa. The major differences expected to impact functionality are displayed by the eIF4G/interacting protein (IP) binding site and the change from a positive to an uncharged residue at Lys-162 using murine residue numbering.

eIF4E-1, but not eIF4E-2 family members can be pulled down using an m^7GTP cap analogue by column chromatography: Only the four eIF4E-1 family members stayed bound to the beads, while eIF4E-2b and the negative control (luciferase) were found mainly in the unbound and wash fractions (Figure 2). These results were confirmed by scintillation counts from the flow through and bead bound fractions. The results from SDS-PAGE and subsequent autoradiography confirmed that each eIF4E was being expressed at the correct molecular weight and it matched the results of the scintillation counts from each fraction. While these results are not a quantitative affinity measurement of a cap binding protein for the mRNA cap, they provide a qualitative assessment of their ability to interact with the m^7GTP cap structure. Table 1 shows that there is a key difference, between the eIF4E-1 and eIF4E-3 family members versus the two eIF4E-2 members, in a residue shown to be important for neutralizing the phosphate bridge of the cap structure, Lys-162.

Table 1. There are eight eIF4E-1 family members, two eIF4E-2 members, and four eIF4E-3 members in the transcriptome of *Karlodinium veneficum*. The five members used for this study are highlighted in bold lettering. The eIF4G/interacting protein site has been extracted and highlighted to show negatively charged residues in red and positively charged residues in blue. In addition, two residues shown to be important for cap interaction are shown, Arg-157, and Lys-162 using murine eIF4E numbering. Both eIF4E-2 family members possess an uncharged valine in place of a positively charged residue at position 162.

eIF4E Family Members	Amino Acid Length	Molecular Weight (kD)	Calculated pI	Transcript Abundance (FPKM)	eIF4G/IP Binding Site	Phosphate Bridge: ARG157	Phosphate Bridge: LYS162
eIF4E-1a1	226	25.9	5.91	4.1	TVQEFW	R	R
eIF4E-1a2	226	25.9	5.79	7.2	TVQEFW	R	R
eIF4E-1a3	226	26.0	5.96	9.4	TVQEFW	R	R
eIF4E-1b1	219	24.5	5.74	9.7	SAQEFW	R	R
eIF4E-1b2	235	26.1	5.97	3.8	TVQEFW	K	R
eIF4E-1c	212	23.7	4.89	8.3	TVEAFW	R	R
eIF4E-1d1	233	26.6	5.93	11.1	TVKEFW	K	R
eIF4E-1d2	237	26.2	6.87	2.6	TVKGFW	K	R
eIF4E-2a	297	33.9	4.97	4.9	TVEGFY	K	**V**
eIF4E-2b	205	23.8	5.73	3.1	TIEQFF	R	**V**
eIF4E-3a1	332	36.9	5.68	3.1	TVEDYW	R	K
eIF4E-3a2	246	27.2	5.39	6.8	TVEDFW	R	K
eIF4E-3b	901	99.4	5.49	3.1	SVEELW	K	K
eIF4E-3c	258	28.8	6.18	5.6	TIEDLW	R	K

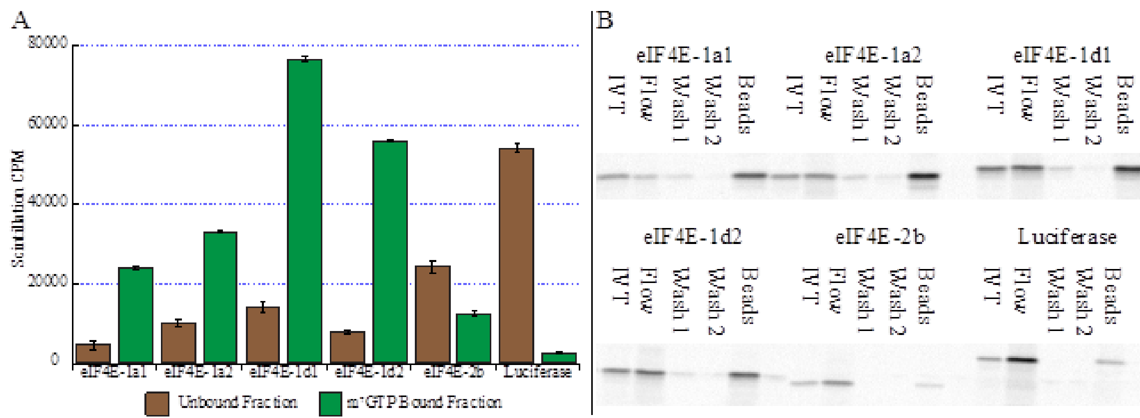

Fig. 2. Each eIF4E-1 family member binds to the m^7GTP cap by column chromatography, while the eIF4E-2 family member does not . **A.** The scintillation counts of unbound and bound fractions were assessed for *K. veneficum* eIF4E family members compared to a negative control, luciferase. *K. veneficum* eIF4E-2b had the lowest binding capacity for the m^7GTP cap column compared to the eIF4E-1 family members. **B**. Autoradiography was used to confirm the presence of an appropriately sized band in each fraction.

In the two eIF4E-2 family members an uncharged valine residue has replaced this lysine. This may explain its lack of strong interactions with cap analogue. From our previous phylogenetic analysis, we made predictions based on amino acid alignments and comparisons to eIF4E crystal structures (Jones *et al.*, 2015). Interestingly, despite the large sequence divergence and evolutionary distance from any other studied eIF4E protein, we were still able to find both a set of cap binding proteins and eIF4E-like proteins that may serve regulatory roles in gene expression. Our preliminary hypothesis is that the eIF4E-1 family members may function as the cap-binding initiation factors, while the eIF4E-2 (and perhaps eIF4E-3) members may serve regulatory roles in gene expression for the dinoflagellates. It is possible that these regulatory roles consist of mRNA sequestration or differential recruitment of other translation initiation factors, for example eIF4G, in response to a particular stimulus. This regulatory method would allow for the continuous presence of mRNA without the necessity for protein expression.

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Surveillance and Management

Shellfish monitoring for lipophilic phycotoxins in France, recommendation for an updated sampling strategy

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Abstract

In France, the sampling strategy for the official monitoring of lipophilic phycotoxins in bivalve shellfish relies on the definition of risk areas and high risk periods, during which a systematic weekly analysis of toxins in shellfish is performed. Since 2010, high risk periods are defined as follows: the occurrence of one result above the European regulatory limit (160 µg equivalent okadaic acid/kg shellfish) over the last 3 years leads to that month being considered a high risk period. This definition was established according to a statistical analysis of the official monitoring results for the period 2003-2008, based on the mouse bioassay (MBA) as the official analytical method. As of the 1st January 2010, the MBA has been replaced by LC-MS/MS. In 2014, a new statistical analysis was performed, based this time on results for the period 2010-2013 for which quantitative LC-MS/MS data are available. We tested the robustness of the definition set in 2010 and identified a new methodology to improve our sampling strategy for lipophilic toxins in bivalve shellfish, based on Bayesian inference.

Keywords: monitoring; shellfish; lipophilic phycotoxins; Bayesian inference

Introduction

The sampling strategy for the official environmental monitoring of phycotoxins in bivalve shellfish in France differs according to the family of toxins and to the type of zone (coastal or offshore). For PSP and ASP toxins, the strategy relies on the monitoring of phytoplankton in seawater. The detection of toxic species above an alert threshold acts as a trigger for the analysis of toxins in shellfish. For lipophilic toxins, the phytoplankton is not a reliable indicator. A systematic weekly analysis of toxins in shellfish is performed in risk areas during high risk periods, firstly proposed in 1999 by Ifremer (the French Research Institute for Exploitation of the Sea). Outside these high risk periods, the strategy relies on phytoplankton analysis, which is the method used for ASP and PSP. The methodology to identify high risk periods for lipophilic toxins has been reviewed in 2010. The occurrence of one result above the European regulatory limit (160 µg equivalent okadaic acid/kg shellfish) over the last 3 years in the area leads to that month being considered as a high risk period in this area. An area with at least one month as risk period is considered as a risk area. This definition has been recommended by ANSES (the French Agency for Food, Environmental and Occupational Health & Safety) based on a statistical analysis of the official monitoring results for the period 2003-2008. At that time, the mouse bioassay (MBA) was the official analytical method but as of the 1st January 2010, it has been replaced by LC-MS/MS. In 2014, ANSES carried out a new statistical analysis, based this time on results for the period 2010-2013 for which quantitative LC-MS/MS data are available (and not only qualitative positive/negative results from the MBA). The objective was to evaluate the performance of the current definition of high risk periods and to look for a new definition that could improve the efficiency of the sampling strategy.

Material and Methods

Based on the data for the 3-year period 2010-2012, we compared the predicted high risk periods to actual periods in 2013 (reference year) with results above the EU regulatory limit and evaluated the sensitivity and the specificity:
- for the current system: based on qualitative data (result above the EU regulatory limit during the last 3 years? YES/NO)
- and for an alternative system: based on quantitative data, evaluation of the probability of

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the area/period to have 1 result > EU regulatory limit. The data analysis involved:

- Lognormal fitting of the data taking into account censoring (by cumulative distribution function)
- Testing 2 models: 1) maximum of likelihood (fitdistrplus package, R.3.03) 2) Bayesian inference (package rjags): higher number of situations can be fitted with this model compared to the maximum of likelihood (e.g. only 1 data available for the area/period), uncertainty is function of the number of analysis done, but interpretation of uncertainty is not always easy.
- The risk manager setting the acceptable level above which the area/period is considered as being at high risk (p-value).

A description of the data used in the analysis is as follows:
- Years: 2010, 2011, 2012, 2013
- LC-MS/MS analysis
- Concentration in shellfish meat, sum of AO+DTXs+PTXs
- based on EFSA toxic equivalence factors
- Number of monitored marine areas: 77
- Number of data (measured concentration): 5 434
- Minimum: 3 µg eq OA/kg shellfish meat
- Maximum: 37 296 µg eq OA/kg shellfish meat
- Number of censored data (< limit of detection): 2 962.

Results and Discussion

Figure 1 illustrates the diversity in the data available according to the shellfish area. For some areas there is a lot of data all year around whereas for other there is data for only 2 or 3 months.
Table 1 shows the high risk periods according to the current system in some shellfish areas for 2013. In high risk periods, there is a weekly sampling of shellfish in the marine area for lipophilic toxins testing by LC-MS/MS. Outside these periods (not high risk periods), there is a water sampling every 2 weeks for plankton monitoring. If the number of cells of *Dinophysis* is above an alert level, there is a shellfish sampling in week n+1 for toxin testing.
In addition to this strategy, for 10 areas (part of the vigilance system), there is a systematic monthly sampling of shellfish all year.

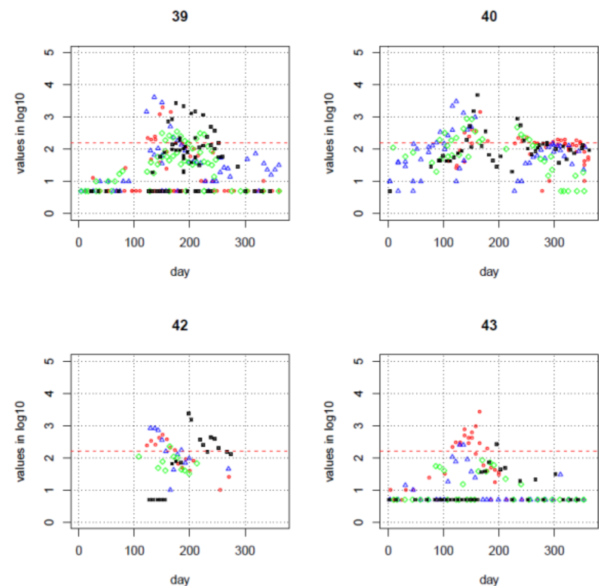


Fig. 1: An illustrative example of toxicity data (y axis: µg OA/kg) for the period 2010-2013 in some areas (39, 40, 42, 43) by day of year.
Legend. 2010: red, 2011: blue, 2012: green, 2013:black, horizontal red dot line: EU regulatory limit.

Table 1: Current system with high risk periods for 2013 by area and month (for areas #3 to 57 as an example). Legend. ND: no data in 2010-2012, by default defined as not a high risk period, 0: defined not a risk period with data, 1: defined as high risk period.

month / area	1	2	3	4	5	6	7	8	9	10	11	12
3	0	0	0	0	0	ND	ND	ND	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
9	ND	ND	ND	ND	ND	0	ND	0	0	0	0	0
10	0	0	0	0	1	1	0	1	1	1	0	0
12	0	0	ND	ND	0	ND	ND	ND	0	0	0	0
13	0	0	0	ND	ND	ND	ND	ND	ND	0	0	0
14	ND	ND	0	ND	0	0	ND	0	0	0	0	0
15	0	ND	0	ND	0	0	0	0	0	0	0	0
16	0	0	0	0	ND	ND	ND	ND	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	ND	ND	ND	ND	ND	0	ND	0
24	0	0	0	0	ND	ND	ND	ND	0	0	0	0
26	ND	ND	ND	ND	0	ND	ND	ND	ND	ND	ND	ND
32	ND	ND	ND	0	1	0	0	0	1	1	0	ND
33	0	0	0	0	ND	0	ND	ND	0	0	0	0
34	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
37	ND	ND	0	1	1	ND	ND	0	0	ND	0	0
38	0	0	ND	1	1	1	0	1	1	1	1	0
39	0	0	0	0	1	1	1	1	0	0	0	0
40	0	0	0	1	1	1	0	1	1	1	1	1
42	ND	ND	ND	0	1	1	1	ND	0	ND	ND	ND
43	0	0	0	1	1	1	0	0	0	0	0	0
44	ND	ND	ND	0	1	1	1	0	ND	ND	ND	ND
45	ND	ND	ND	0	1	1	0	ND	ND	ND	ND	ND
46	ND	ND	ND	0	1	1	0	ND	ND	ND	ND	ND
47	0	0	0	1	1	1	1	1	0	0	0	0
48	ND	ND	ND	0	1	1	0	ND	ND	ND	ND	ND
49	0	0	0	1	1	1	1	0	0	0	0	0
50	ND	ND	ND	ND	0	ND	ND	ND	ND	ND	ND	ND
51	ND	ND	ND	ND	1	1	1	ND	ND	ND	ND	ND
52	ND	ND	ND	ND	1	1	ND	0	0	ND	ND	ND
53	ND	ND	ND	0	1	1	0	0	0	0	ND	ND
54	0	0	0	0	0	0	0	0	0	0	0	0
55	ND	ND	ND	ND	0	0	0	ND	ND	ND	0	ND
56	ND	ND	ND	ND	0	ND	ND	ND	ND	0	ND	ND
57	ND	ND	ND	ND	0	0	ND	ND	ND	0	ND	ND

Table 2: Comparison of observed data and predicted high risk periods in 2013 (for areas #3 to 57 as an example).

month / area	1	2	3	4	5	6	7	8	9	10	11	12
3	0	0	0	0	0	NA	NA	0	0	2	0	0
6	0	0	0	0	0	0	0	0	NA	0	0	0
9	0	0	0	0	NA	NA	NA	0	0	2	0	0
10	0	0	0	0	1	1	0	3	3	3	0	0
12	NA	NA	NA	NA	NA	NA	NA	0	NA	NA	0	0
13	0	0	NA	NA	NA	NA	NA	0	NA	NA	0	0
14	0	NA	NA	NA	NA	NA	NA	0	NA	NA	1	1
15	0	0	NA	NA	NA	0	0	0	0	0	0	NA
16	0	0	0	0	NA	NA	NA	NA	0	0	0	0
18	0	0	0	0	0	0	0	0	0	0	0	
21	0	0	0	0	NA	NA	NA	NA	NA	NA	NA	0
24	0	0	0	0	NA	NA	NA	NA	0	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0	0
32	NA	NA	NA	0	1	0	0	0	1	1	0	NA
33	0	NA	NA	NA	NA	0	0	0	0	0	0	0
34	NA	NA	NA	NA	NA	NA	0	NA	NA	NA	NA	NA
37	NA	NA	0	1	-1	NA	0	0	NA	NA	NA	NA
38	0	0	0	1	1	3	0	3	3	1	1	0
39	0	0	0	NA	1	3	3	3	2	0	0	0
40	0	NA	0	1	3	3	0	3	3	1	1	1
42	NA	NA	NA	NA	1	1	3	2	2	0	NA	NA
43	0	0	0	1	1	1	2	0	0	0	0	0
44	NA	NA	NA	NA	1	1	3	2	0	NA	NA	NA
45	NA	NA	NA	NA	1	1	2	NA	NA	NA	NA	NA
46	NA	NA	NA	NA	1	1	2	0	0	0	NA	NA
47	0	0	0	1	1	3	3	3	2	6	6	1
48	NA	NA	NA	NA	1	1	2	NA	0	0	NA	NA
49	0	0	0	1	1	3	3	0	0	2	0	NA
50	NA	NA	NA	NA	NA	NA	0	NA	NA	0	NA	NA
51	NA	NA	NA	NA	1	1	3	NA	NA	0	NA	NA
52	NA	0	NA	NA	-1	-1	NA	NA	NA	NA	NA	NA
53	NA	NA	NA	NA	1	1	NA	NA	NA	NA	NA	NA
54	0	0	0	0	0	2	2	0	0	0	0	0
55	NA	NA	NA	NA	NA	NA	NA	0	NA	0	NA	NA
56	0	0	0	0	0	0	0	0	0	0	0	0
57	0	0	0	0	0	0	0	0	0	0	0	0

Legend:
Green 3: true positive predicted (TPP): result > EU regulatory limit in 2013 and predicted as high risk period
white 0 : true negative predicted (TNP): no result > EU regulatory limit in 2013 and predicted as not high risk period
red 2: false negative predicted: result > EU regulatory limit in 2013 but NOT predicted as high risk period
blue 1: false positive predicted: no result > EU regulatory limit in 2013 but predicted as high risk period.
NA: no data.

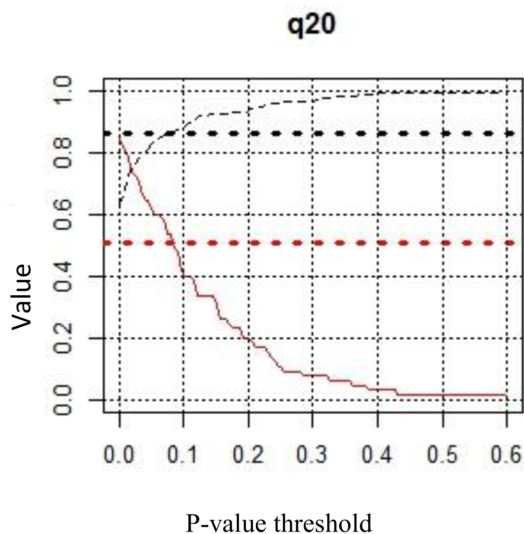
Table 2 shows the comparison of observed data and predicted high risk periods in 2013. Table 3 shows the proposed new systerm, based on Bayesian inference and providing the probability (p-value) of results above the EU regulatory limit by marine area and month for 2013, based on all available quantitative data in 2010, 2011 and 2012. The prior is a probabilistic distribution reflecting the level of knowledge we have before taking into account the data. In this study we chose a non-informative prior probabilistic distribution for estimating the risk for 2013 before adding the data (2010-2012), setting that we assume to know quite nothing about the area and the period without the knowledge of the data. Then, the inference is only linked by the data and the prior is not supposed to influence the results. After making inference with the data set, we estimate the risk for each area and period.The risk manager sets the acceptable level (p-value, e.g. 10%, 5%, 1%) and identifies high risk periods.

Table 3: probability (p-value) of results above the EU regulatory limit by marine area and month for 2013, based on Bayesian inference (new system). ND, NA: no data.

month / area	1	2	3	4	5	6	7	8	9	10	11	12
3	NA	NA	0,00	NA	NA	ND	ND	ND	0,00	0,00	0,00	0,01
6	0,00	NA	0,00	NA	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
9	ND	ND	ND	ND	ND	NA	ND	0,00	0,01	0,00	0,03	0,01
10	0,00	0,00	0,00	0,00	0,07	0,10	0,01	0,17	0,15	0,12	0,08	0,04
12	NA	NA	ND	ND	0,00	ND	ND	ND	0,00	0,00	0,01	0,01
13	0,05	0,00	0,00	ND	ND	ND	ND	ND	ND	0,00	0,01	NA
14	ND	ND	0,00	ND	0,00	0,00	ND	NA	0,00	0,00	0,00	0,00
15	0,00	ND	0,00	ND	NA	NA	0,00	0,00	0,00	NA	NA	NA
16	0,00	0,00	NA	NA	ND	ND	ND	ND	0,00	NA	0,00	NA
18	0,00	NA	0,00	NA	0,00	NA	NA	NA	NA	0,00	NA	NA
21	NA	NA	NA	NA	ND	ND	ND	ND	ND	0,00	ND	0,00
24	0,00	NA	NA	0,00	ND	ND	ND	ND	NA	0,00	0,00	NA
26	ND	ND	ND	ND	0,00	ND	ND	ND	ND	ND	ND	ND
32	ND	ND	ND	0,00	0,11	0,00	NA	0,01	0,11	0,10	0,02	ND
33	NA	0,00	0,00	0,00	ND	0,00	ND	ND	0,00	0,00	NA	0,00
34	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
37	ND	ND	0,00	0,12	0,29	ND	ND	0,00	0,04	ND	0,02	0,00
38	NA	0,00	ND	0,22	0,31	0,23	0,03	0,10	0,17	0,09	0,12	0,00
39	0,00	0,00	0,01	0,00	0,23	0,15	0,15	0,08	0,02	0,01	0,02	0,01
40	0,05	0,10	0,10	0,43	0,43	0,69	0,12	0,20	0,19	0,12	0,21	0,12
42	ND	ND	ND	0,13	0,65	0,23	0,02	ND	0,03	ND	ND	ND
43	0,00	0,00	0,02	0,09	0,30	0,24	0,04	0,00	NA	0,00	0,01	0,00
44	ND	ND	ND	0,02	0,22	0,31	0,07	0,01	ND	ND	ND	ND
45	ND	ND	ND	NA	0,10	0,08	0,04	ND	ND	ND	ND	ND
46	ND	ND	ND	0,03	0,25	0,12	0,12	ND	ND	ND	ND	ND
47	0,02	0,01	0,00	0,09	0,33	0,36	0,21	0,09	0,03	0,00	0,00	0,01
48	ND	ND	ND	0,02	0,21	0,17	0,05	ND	ND	ND	ND	ND
49	0,00	NA	0,00	0,10	0,29	0,29	0,19	0,03	0,02	0,02	0,00	0,00
50	ND	ND	ND	ND	0,07	ND	ND	ND	ND	ND	ND	ND
51	ND	ND	ND	ND	0,22	0,20	0,08	ND	ND	ND	ND	ND
52	ND	ND	ND	ND	0,21	0,20	ND	0,08	0,03	ND	ND	ND
53	ND	ND	ND	NA	0,14	0,11	0,10	NA	NA	NA	ND	ND
54	NA	0,00	0,00	0,00	0,05	0,12	0,09	0,01	0,05	0,02	0,03	0,01
55	ND	ND	ND	ND	0,02	0,01	0,00	ND	ND	ND	0,00	ND
56	ND	ND	ND	ND	0,04	ND	ND	ND	ND	0,01	ND	ND
57	ND	ND	ND	ND	0,04	0,02	ND	ND	ND	0,02	ND	ND

The current system of definition of high risk periods (1 result > EU regulatory limit over the last 3 years) has a sensitivity of 51% and a specificity of 86%.
Criteria definition :
- Sensitivity: probability (predicted+/found +) = TPP/(TPP+False Negative)
- Specificity: probability (predicted-/found-) = TNP/(TNP+False Positive)
+: above the EU regulatory limit of 160 µg eq OA/kg shellfish meat
-: below

In the proposed new definition of high risk periods the sensitivity and the specificity are function of the acceptable level (as shown in Figure 2). Sensitivity can reach 85% (Bayesian inference, p-value of 0.1%).



P-value threshold

Fig. 2. Sensitivity and specificity of the proposed new system.
Legend:
red line: sensitivity
black line: specificity
dotted red line: sensitivity of the current system
dotted black line: specificity of the current *system*

However, this improvement in terms of sensitivity and specificity implies an increase in the number of shellfish samples necessary (an increased number of high risk periods), as shown in table 4. The prior we used was the same for all months and areas. However it could be feasible to set informative priors, different for some areas or months based on environmental or historical data, and then to better identify at-risk areas.

Conclusion

The strengths of the new system (Bayesian inference is the preferred option) include 1) the use of all the data information (quantitative) and not only a part of it (qualitative), 2) the ability to fit more situations (e.g. when there is only 1 result for a month with a value below, but close to, the EU regulatory limit), 3) the possibility for increased sensitivity, and therefore a better protection of consumer health, 4) the possibility to take into account extra information in the prior, 5) an increased role of the risk manager who can choose the level of protection.

As drawback, this new system implies the need for an increased number of shellfish-meat toxin analysis and consequently, increased costs.
However, this work only relies on one reference year (2013). To confirm our modelling and our recommendations, we plan to conduct a new statistical analysis with the data now availalble for the year 2014. This study is expected to be completed by mid-2015.

Table 4. Sensitivity and specificity of the proposed new system according to the p-value threshold and the numer of high risk periods.

Sensitiviy	P-value threshold	Number of high risk periods	Specificity
0.85	0.10	282	0.63
0.85	0.20	275	0.65
0.83	0.30	271	0.65
0.82	0.70	257	0.67
0.80	1.40	226	0.72
0.78	1.70	217	0.74
0.77	1.80	214	0.74
0.75	2.00	210	0.74
0.74	2.30	199	0.75
0.72	2.90	179	0.78
0.71	3.40	173	0.78
0.68	3.50	170	0.79

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Anses opinion, 29 July 2012 (only available in French). Avis de l'Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail relatif aux modalités de définition des périodes à risque et des zones à risque concernant les phycotoxines lipophiles contaminant les coquillages dans le milieu marin, https://www.anses.fr/sites/default/files/documents/ERCA2012sa0272.pdf

Tracking Algal Changes in the Neuse River Estuary, North Carolina, USA from 1998-2013

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Abstract

In response to harmful algal blooms and fish kills on the Neuse River during the 1990s, a permanent 30 percent reduction in total nitrogen entering the estuary was implemented from late 1998 through 2003. Continuous taxonomic data on phytoplankton assemblages in addition to photic zone chemical (chlorophyll *a*, NO_x^-, total Kjeldahl N [TKN], total P, pH) and physical (temperature) data collected from three locations by the North Carolina Division of Water Resources on the Neuse River from 1998-2013 were examined. Within this construct, this study considers the role of nutrient reduction regulations on populations of three harmful algal bloom taxa—the cyanobacteria *Cylindrospermopsis raciborskii* and the dinoflagellates *Karlodinium veneficum* and *Prorocentrum minimum* within the Neuse River before and after nutrient reductions were implemented. While, in general, the data suggest that there were few changes in *K. veneficum* and *P. minimum* abundance before and after nutrient reductions, there were significant increases in *C. raciborskii* abundance in all three locations. Moreover, all three species increased in frequency of occurrence at one or more locations after 2003. Water quality data also show a significant decrease in NO_x^- at two sites and significant increases in TKN at all locations after 2003.

Keywords: Cyanobacteria, Dinoflagellates, Eutrophication, Neuse River, Nutrients.

Introduction

Due to repeated occurrences of algal blooms and high chlorophyll *a* levels, the lower Neuse River estuary was placed on a federal list of impaired waters. In 1996, North Carolina legislation was passed (Sessions Law 1995, Section 572) to implement a 30 percent reduction in total nitrogen entering the estuary and thereby limiting the extent and duration of algal blooms (Lebo *et al*. 2012). An outcome of this legislation was a comprehensive set of rules that began in 1998 and fully implemented by 2003. To date, little attention has been given to understand how these nutrient reduction regulations have impacted algal blooms in the Neuse River Estuary. Therefore, the purpose of this study was to compare changes in nutrient levels and algal populations before and after nutrient reductions. Specifically, we wanted to consider the populations of three harmful algal bloom taxa within the lower Neuse River - the cyanobacteria *Cylindrospermopsis raciborskii* and the dinoflagellates *Karlodinium veneficum* and *Prorocentrum minimum.*

Materials and Methods

Environmental data were collected (Jan. 1998 to Dec. 2013) as part of a routine monitoring program conducted monthly to semi-monthly by the North Carolina Division of Water Resources (NCDWR). Nutrients collected included NO_x^-, total Kjeldahl nitrogen (TKN), and phosphorus (P). Subsurface physiochemical measurements of pH and temperature were also recorded using a Hydrolab Surveyer-4 (Hydrolab Corp., Austin, Texas, USA). Chlorophyll *a* concentrations were assayed using standard spectrophotometric procedures. Phytoplankton samples were sampled within the photic zone (defined by NCDWR as twice the Secchi depth), preserved in the field with acidic Lugol's solution, and stored on ice (~4°C) during transit to the laboratory. Total microplankton greater than 5 μm were enumerated using an Utermöhl settling chamber under 300X magnification on an inverted Leitz Diavert microscope. Phytoplankton populations were reported as cells (*K. veneficum* and *P. minimum*), or filaments (*C. raciborskii*). Biological and environmental data were classified and pooled

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into two groups according to when the sample was collected. These classifications included 'pre-reductions' (samples collected prior to Dec. 31, 2003) and 'post-reductions' (samples collected after Jan.1 2004). Because the data was often not normally distributed, a Kruskal-Wallis one-way ANOVA was employed to statistically compare pre- and post-reduction values for algal abundance and water quality parameters. We also considered the frequency in which each algal species occurred (pre- and post-reductions). This data was analyzed using a parametric one-way ANOVA. All tests were considered statistically significant at an $\alpha < 0.05$.

Results and Discussion

Although there were significant declines in water-column NO_x^- post 2003 in two of the three sites (Fig. 1e; $p \leq 0.009$), there was an overall increase in total Kjeldahl nitrogen (TKN) at all three locations ($p \leq 0.025$; Fig 1d). This higher organic nitrogen suggests that water-column productivity may be increasing -fostering greater demands for N and a concomitant decrease in inorganic N (including NO_x^-) within surface waters.

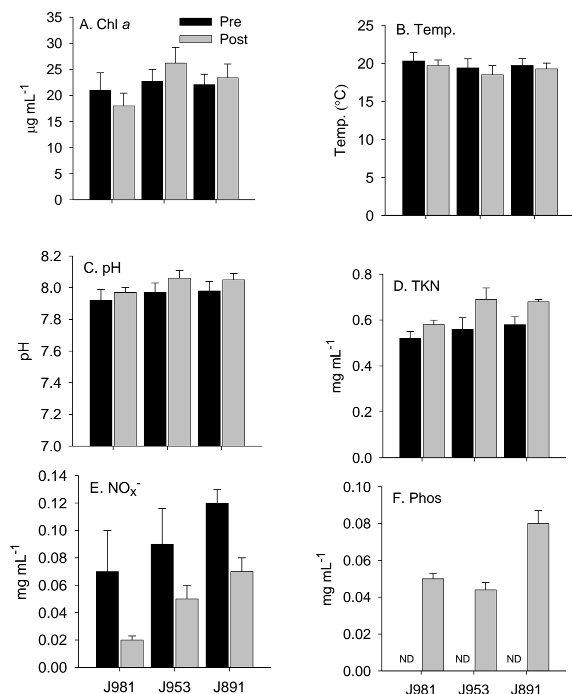

Fig. 1. Water quality parameters before- (black) and after- (gray) 2003 regulations. Data include three sites evaluated in this study (J981, J953, and J891). ND signifies not determined, asterisks indicates significant differences, and data are presented as means ± 1 standard error (SE).

As for specific algal species, no significant changes in cell density were observed for *Prorocentrum minimum* at any site ($p \geq 0.175$; Fig. 2a). However, there was a significant increase in *P. minimum* occurrence (i.e., proportion of samples containing *P. minimum*) at site J953 after 2003 (p=0.033; Fig. 3). In this case, average annual occurrence rate increased from 27.3 ± 3.6 to 44.0 ± 5.8 % of samples collected from pre- to post-regulations, respectively.

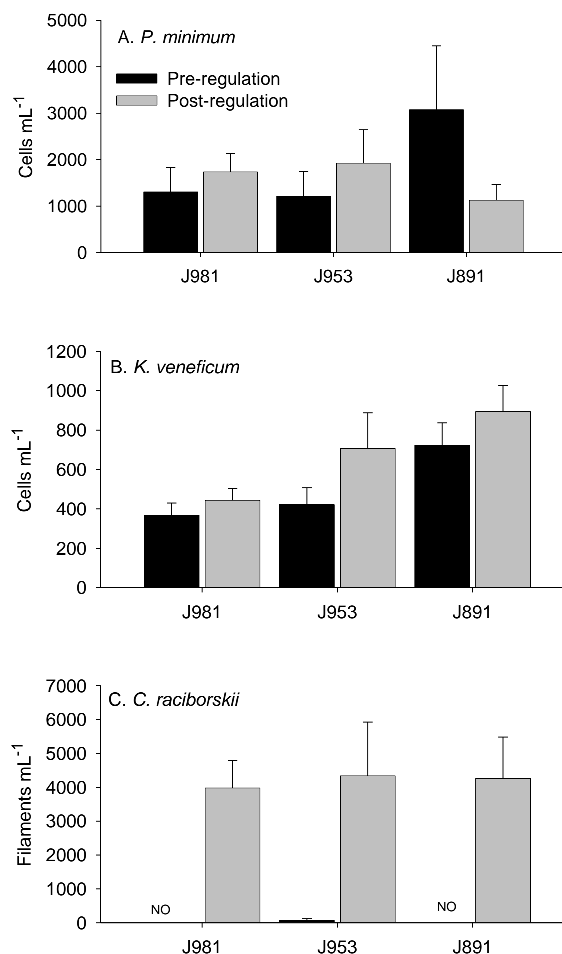

Fig. 2. Algal abundance before and after 2003 for *P. minimum* (A), *K. veneficum* (B), and *C. raciborskii* (C) collected at three locations (J981, J953, and J891). NO indicates not observed. Data are presented as means ± 1 SE.

Similarly, there were no significant differences in abundance for *Karlodinium veneficum* before and after N regulations in the Neuse River estuary ($p > 0.08$; Fig. 2b). However, as with *P. minimum*, there was a significant increase in occurrence of *K. veneficum* after 2003 in site J953 ($p = 0.041$; Fig. 4b). For this location, *K. veneficum* increased from 45.0 ± 6.3 to 62.4 ± 1.8 % when comparing

between pre- and post-regulations, respectively. In contrast, there were significant increases in *Cylindrospermopsis raciborskii* abundance ($p < 0.001$; Fig. 2) and frequency of occurrence at all

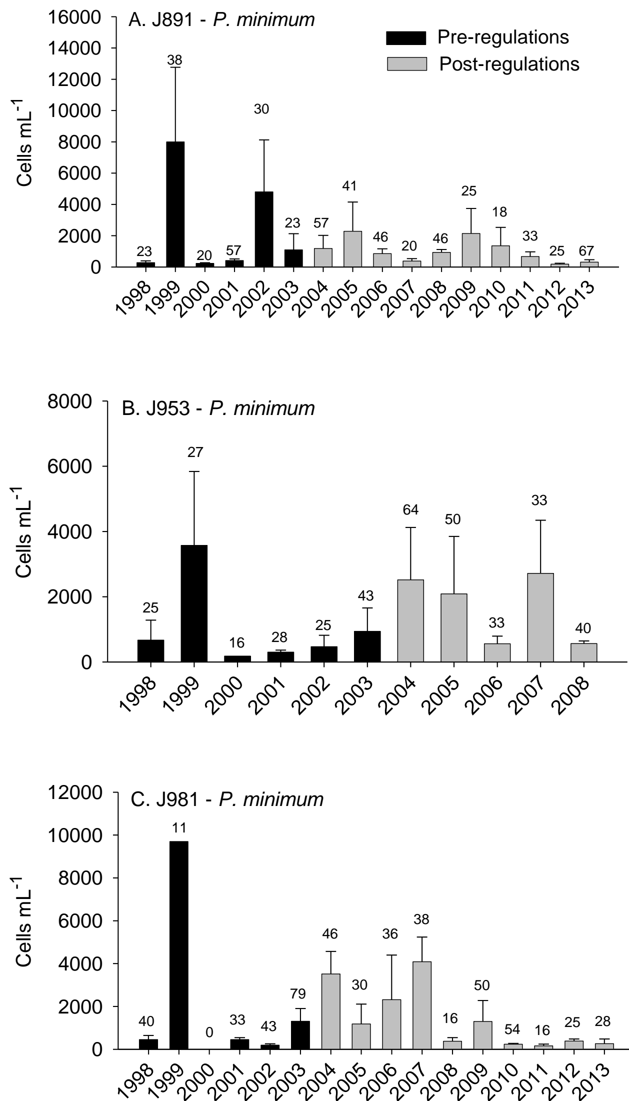

Fig. 3. *P. minimum* abundance for each year sampled at three locations (J891 [A], J953 [B], and J981 [C]). Number above bar indicates percent of the samples containing *P. minimum*. Data are presented as means ± 1 SE.

three locations in the lower Neuse River estuary after 2003 ($p \leq 0.033$; Fig. 5). Indeed, prior to 2003, *C. raciborskii* was only observed at site J953 - where it was recorded in 18% of the samples with a mean density of only 70 ± 50 filaments mL^{-1} in 1999 (Fig. 5). This agrees with other studies that have observed increased *C. raciborskii* abundances and expansion in mid-latitudes driven, in part, by its phenotypic plasticity (Briand *et al.* 2004; Bonilla *et al.* 2012).

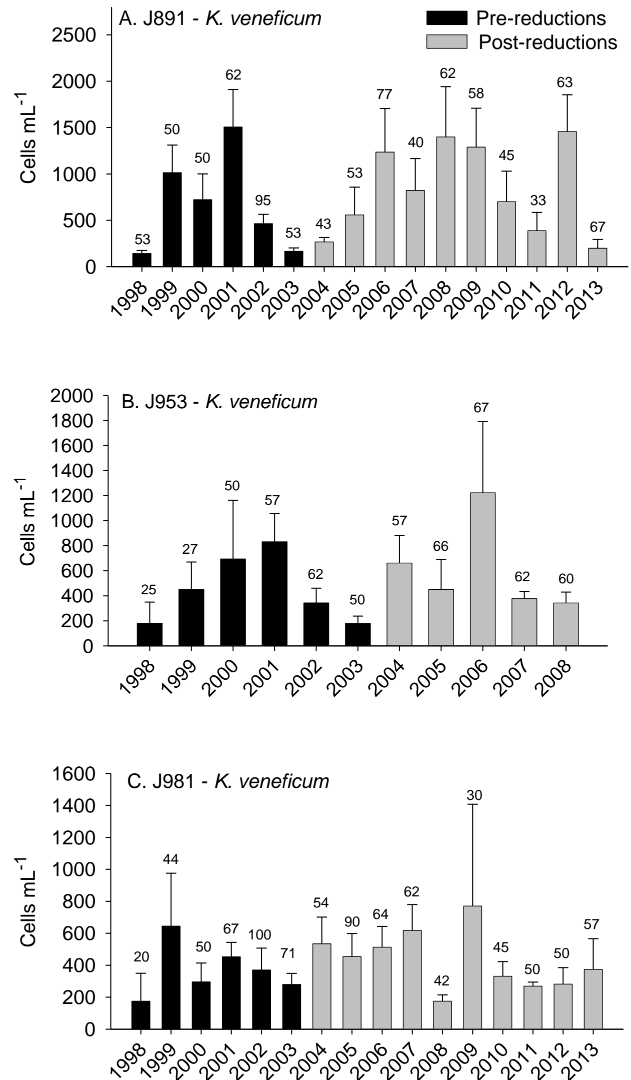

Fig. 4. *K. veneficum* abundance for each year sampled at three locations (J891 [A], J953 [B], and J981 [C]). Number above bar indicates percent of the samples containing *K. veneficum*. Data are presented as means ± 1 SE.

Although we observed few changes in *K. veneficum* and *P. minimum* abundance before and after nutrient loading reductions, and significant increases in *C. raciborskii* abundance in all three locations, this does not necessarily indicate that nutrient reduction policies are ineffective over longer periods. For example, other factors may play a role in how quickly phytoplankton respond to nutrient reductions including system morphology, residence time, sediment type, trophic state, zooplankton grazing, and history of enrichment (Marsden 1989; Carvalho and Kirika

2003; Jeppesen *et al*. 2005). Moreover, in a Danish study involving a 50% reduction in N and an 80% reduction in P for 14 years, it was

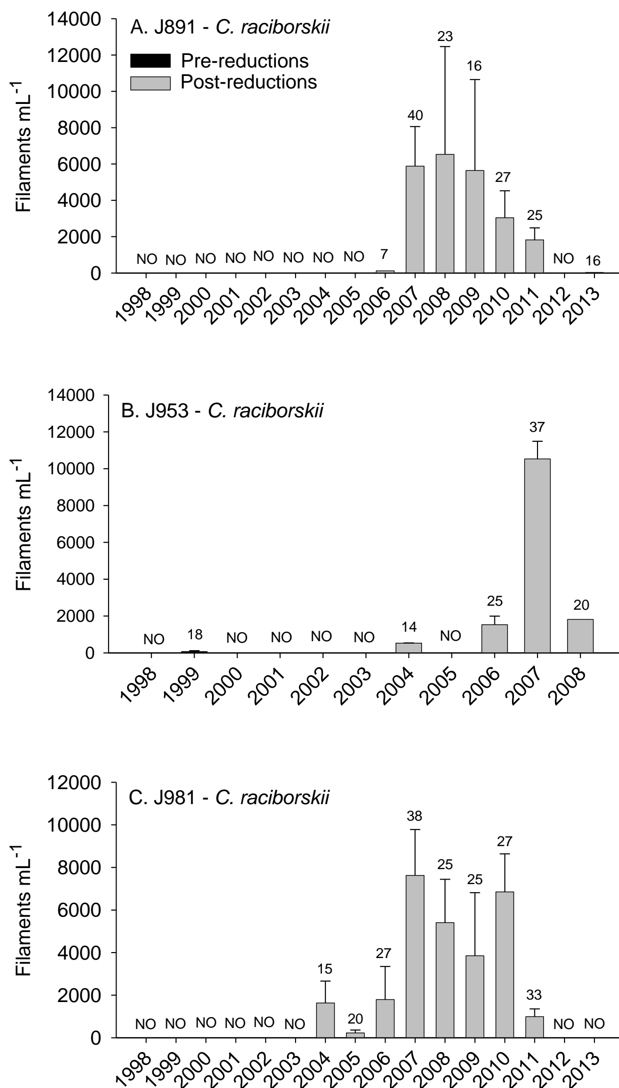

Fig. 6. *C. raciborskii* abundance for each year sampled at three locations (J891 [A], J953 [B], and J981 [C]). Number above bar indicates percent of the samples containing *C. raciborskii*. NO indicates that the alga was not observed during that year. Data are presented as means ± 1 SE.

demonstrated that estuaries typically responded much slower to nutrient reductions than other systems (e.g., freshwater streams and lakes; Kronvang *et al.* 2005). Therefore, complex estuarine systems such as the Neuse River, with a history of high nutrient enrichment, may take several decades before significant reductions harmful algae and more generally phytoplankton abundance is observed.

Acknowledgements

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The New York State citizen-based monitoring program for cyanobacteria toxins

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Abstract

In 1985, New York State Department of Environmental Conservation and the New York State Federation of Lake Associations established a citizen-based monitoring program to track lake water quality throughout New York State. Starting with 25 lakes and 100 volunteers, the Citizens Statewide Lake Assessment Program (CSLAP) has grown to include more than 240 lakes and 1,500 volunteers since its inception. Volunteers were trained in sampling techniques and collected samples biweekly between May and October. This program expanded in 2011 to include cyanobacteria and their toxins: microcystins, anatoxin-a, β-methyl -aminoalanine and cylindrospermopsins. The bloom and open water samples collected by citizen volunteers were quickly analyzed to determine cyanobacteria abundance, then analyzed for common cyanobacterial toxins using a combination of enzyme inhibition assays (PPIA) and analytical approaches (LCMS and LC-MS/MS). Presence-absence information was posted on the NYSDEC website for public consumption. In addition to providing a rapid monitoring and alert protocol for the protection of lake users from harmful cyanobacterial blooms, information from CSLAP is used for lake assessment, to develop state-wide nutrient criteria for lake remediation, and to determine the cyanotoxins of most concern in New York State.

Keywords: Citizen, lake, monitoring, cyanobacteria, toxins, microcystins.

Introduction

The New York State Citizens Statewide Lake Assessment Program (CSLAP) started in 1985 when the New York State Department of Environmental Conservation (NYSDEC) proposed using trained volunteers to collect information on the State's water bodies. This citizen-based monitoring program was developed to identify problems and water quality trends, educate the public about lake stewardship, and provide the state with a cost effective means to effectively manage and protect its valuable water resources. The 25 lakes included in the initial pilot program were solicited from the New York State Federation of Lake Associations (NYSFOLA) membership and included a mixture of private and public lakes; lake associations, fish and game clubs, and park districts; small ponds and large lakes; and lakes selected from across the entire geographical range of state. This pilot program included training and the provision of essential sampling equipment to the participating volunteers. The samples collected were shipped by post to certified labs for the analysis for nutrients and other water quality parameters. Since its origin, the CSLAP program has rapidly grown to include more than 240 lake associations. The NYSDEC implemented a "5 years on, 5 years off" rotation in 1994 to control rising costs. This led to a slight drop in the number of participating lake associations that year (Figure 1). In 2002, NYSDEC switched from using the New York State Department of Health (NYSDOH) to the private Upstate Freshwater Institute (UFI) as its analytical laboratory. CSLAP instituted a small participation fee which also led to some of the smaller lake associations leaving the program. Other lake association have joined program as its value has become better recognized. CSLAP now has approximately 120 lake associations that participate in any given year (Figure 1).

Initially, the CSLAP program was started to monitor for changes in water quality and hence focused heavily on traditional limnological parameters. Samples were collected from a single mid-lake location and measurements included Secchi depth, air and water temperature, water depth, hypo- and epi-limnetic nutrients (TP, NOx, NH_4 and TDN), specific conductance, pH, water

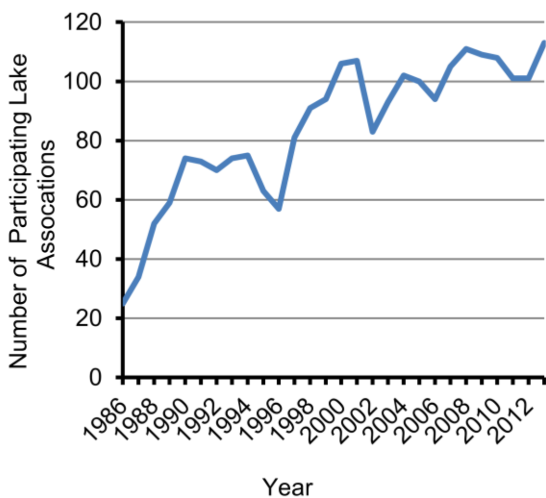


Fig. 1. The number of participating lake associations in CSLAP by year. In most cases, each lake has a single sample site. Multiple sites may be sampled in some of the larger lakes.

color, and chlorophyll-a. Additional parameters have been added or subtracted over the years, and some larger lakes now collect samples from multiple basins. CSLAP volunteers have also provided information on lake perception, invasive species, dreissenid and aquatic plant abundance, ice cover, and shoreline assessments. In 2008, the program was expanded in cooperation with NYSDOH to include cyanobacterial abundance and the presence of microcystin cyanobacterial toxins. Sampling in this initial 1-year effort was limited to periodic late summer sampling in highly eutrophic lakes or potable water supplies. It was limited both spatially and temporally, but confirmed the validity of using trained volunteers for sampling. That led in 2011 to the existing harmful algal blooms (HAB) monitoring partnership between NYSDEC and ESF. The rest of this paper will focus on these recent HAB aspects of the CSLAP program.

Materials and Methods

Each year, volunteers were issued a cooler and ice packs for shipping samples, a set of equipment and supplies for collecting and processing (filtering) samples, and 8 bags of pre-labelled sample bottles with accompanying paperwork. New volunteers underwent training at the NYSFOLA meeting each spring and sample collection generally started in late May depending on the weather conditions. Surface and hypolimnetic grab samples were collected using a Kemmerer bottle biweekly throughout the course of the season. HAB samples were of two types: (1) a 200 ml open water sample collected from the mid-lake location. These samples were filtered in the field onto 0.45µ mixed cellulose ester filters and shipped with the nutrient bottles to UFI for transfer to ESF. (2) An additional 200 ml surface skim sample (aka bloom sample) collected from any location on the lake that the volunteer thought might be experiencing a blue green algae bloom. Bloom samples were mailed directly to ESF for toxin analysis. Upon receipt of the samples, cyanobacterial abundance was determined using a bbe Moldaenke FluoroProbe (Kring *et al.* 2014). Samples with blue green algal chlorophyll (BGA-Chl), as determined using the FP phycocyanin channel, greater than 10 µg/L were visually examined for cyanobacterial using an inverted microscope. A 100 ml subsample was lyophilized to dryness, suspended in 2x5 ml 50% aqueous methanol and the cells lysed by sonication. The resulting lysate was centrifuged to remove particulate material, and the supernatant analysed for cyanobacteria toxins as described below. Filters collected from the mid-lake location were sonicated in 10 ml 50% aq. methanol and then prepared as described above (Boyer 2007).

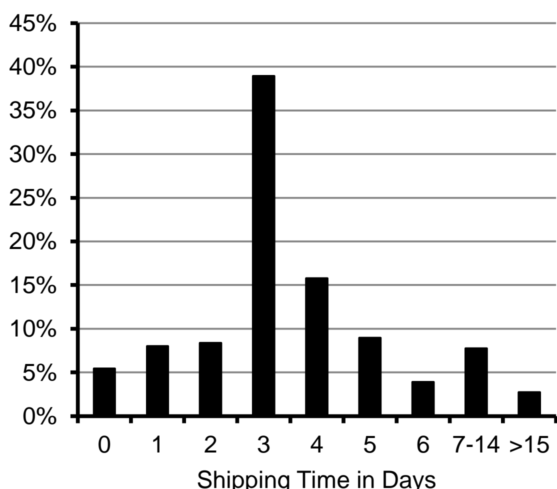
Toxin analysis has been described elsewhere (Boyer 2007). Briefly, microcystins were analysed using the protein phosphatase inhibition assay (PPIA) or by HPLC analysis coupled with photodiode array (PDA) and mass selective (MS) detection. A suite of 14 microcystins (Figure 4) was quantified against microcystin (MC)-LR using the extracted ion corresponding to their molecular weight. Cylindrospermopsin (CYL), epi-CYL, deoxy-CYL, anatoxin-a (ATX) and homo-ATX were analysed by LC-MS or by LC-MS/MS and quantitated against standards of ATX-a or CYL. β-methylaminoalanine (BMAA) was analysed using LCMS, or LC-MS\MS with multiple confirmation ions, using diaminobutryate and BMAA as standards (Boyer, in preparation).

Results and Discussion

The analytical methods adopted for CSLAP HAB monitoring represent a balence of ease of use, cost, speed, sensitivity and suitability for high throughput. CSLAP strives for a 1 week sample turnaround time and thus compromises were developed to meet these requirements. ELISA was too expensive, MALDI-TOF-MS too non-specific, and LC-MS/MS too specific for routine analytical use. PPIA (assay) and LCMS (analysis) offered the best compromise for speed, price,

sensitivity and effort. Use of a single extraction protocol for all toxins (50% aq. methanol with ultrasound: Boyer 2007) was also essential to meet the program requirements.

Samples were collected any day of the week but most often on Saturdays or Sundays. The time between sample collection and receipt by ESF is shown in Figure 2a. Determination of BGA-Chl using the bbe FluoroProbe proved useful for prioritizing samples and the visual analysis of suspect samples was usually completed within 24 hr. The determination of microcystin toxins was completed over the next 3-5 days (Figure 2b); in 2014, 80% of the microcystin analysis were reported back to NYSDEC within the 7-day target period and 90% of the samples were reported back within a 12 day period. Instrument down time, vacations, and planned delays accounted for the delay in the remaining 10% of the samples.



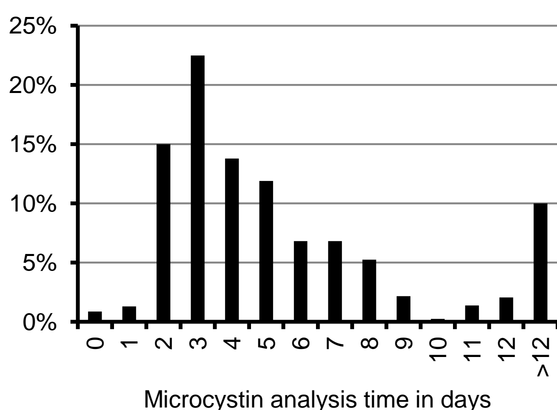


Fig. 2. (a) Time between the sample collection and receipt of the sample by ESF in 2014.

(b) Time between sample receipt and the reporting of analytical results for microcystins to the NYSDEC in 2014 (n = 1170).

Most of the open-water samples received through CSLAP had very low levels of BGA-Chl. In contrast, bloom samples could have very high concentrations of BGA-chl with some samples collected from surface scums exceeding 60,000 µg BGA-Chl per liter (Figure 3).

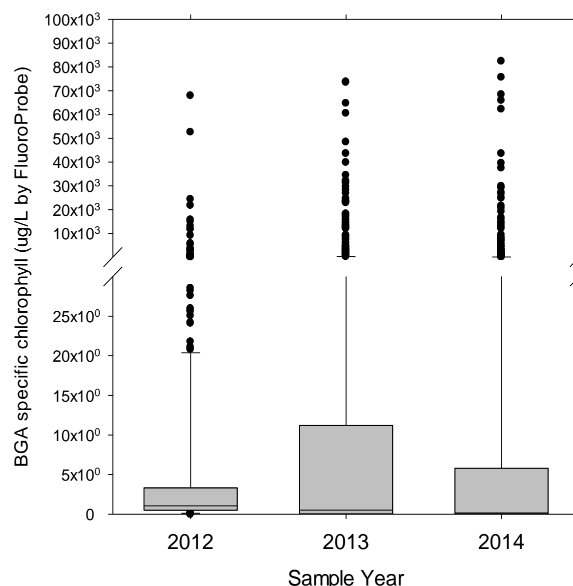


Fig. 3. The distribution of BGA specific chlorophyll in CSLAP samples collected in 2012 (n=826), 2013 (n=1,129) and 2014 (n=1,171). The medium (line), 25-75% quartiles (box), 90 per-centile (whisker) and outliers (dots) are indicated.

As expected, most of the samples collected by the volunteers were non-toxic (Table 1). There were differences in the number of samples with detectable microcystins between years that could not be explained by differences in the detection limits of the two analytical approaches (generally 0.1-0.3 µg/L). Year 2013 had greater maximum toxicity, average toxicity of the top 10 samples, and average toxicity of the top quartile than 2011, 2012. and 2014. These results indicate the high toxin values in 2013 were not isolated events but occurred broadly accross multiple lakes. The high average for the top quartile in 2014 was likely an anomoly due to changing from PPIA to LCMS as the primary analytical method. This change resulted in fewer samples with detectable levels of microcystins between 0.1 and 0.3 µg/L and shifted the position of the quartile. It may also reflect the greater number of bloom samples submitted by

Table 1: Microcystin Abundance in Samples Collected by the CSLAP Volunteers by Year

Sample Year	2011	2012	2013	2014
Total number of samples:	543	891	1157	1171
Samples with detectable Microcystins (%):	184 (34%)	225 (25%)	313 (27%)	76 (6%)
Primarly analytical method	PPIA	PPIA	PPIA	LCMS
Range (µg/L)	0.3-4,080	0.1-3,880	0.2-12,321	0.3-7,712
Average for the top 10 samples (µg/L):	1,200	680	4,200	1,560
Average of the top quartile (µg/L):	300	130	670	840

volunteers in 2014. Hotto (2014) reported the *mcy* B biosynthetic gene was present in ca 50% of sites sampled accross New York State. The lower abundance of microcystins measured using LC-MS or PPIA suggests using the presence of the biosynthetic genes for monitoring microcystins may overestimate the occurrence of the actual microcystin toxin in the system.

Anatoxin-a was also present in 21 samples (1.8%) in 2014, ranging in concentration from 0.1 (detection limit) to >10 µg/L. CYLs and BMAA were not observed in any samples. These latter results are in agreement with the results from CSLAP samples collected in 2011-2013, and with other surveys throughout the New York State that show that microcystins and to a lesser degree ATX-a are the toxins of concern, and that CYLs and BMAA were generally absent (Boyer 2007; Watson *et al,* 2008; unpublished).

Microcystins are a family of 100+ closely related peptides, many with differing degrees of biological activity (Neidermeyer *et al*, 2014). Use of LCMS provided information on the toxin congeners present in New York State (Figure 4). Microcystin LR was the most common congener and was found in almost 90% of the positive CSLAP samples. This was followed by microcystin RR (46%) and YR (37%). Microcytins LA AR, FR, and demethyl derivatives were found in <10% of the samples. Most positive samples had between 1-3 microcystins, though some complex toxin profiles were observed.

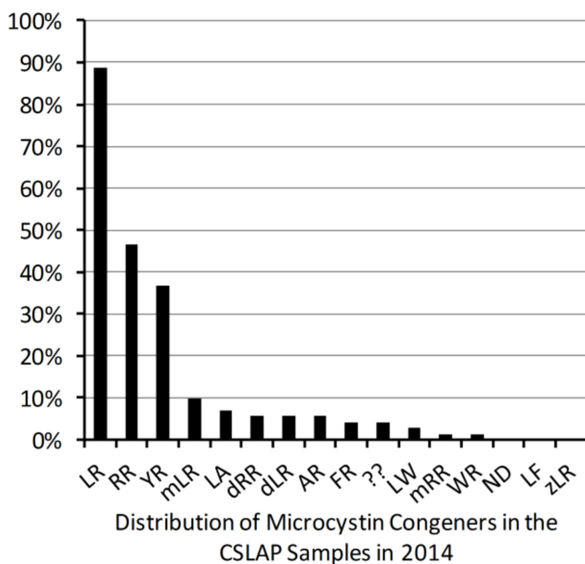


Fig. 4. Distribution of the Microcystin con-geners in the 2014 CSLAP samples.

In summary; New York State has a very cost effective citizen-based HAB monitor system that meets State requirements and provides a rich database of information for HAB researchers.

Acknowledgements

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A summary of harmful algal bloom monitoring and risk assessment within shellfish harvest areas in New South Wales, Australia

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Abstract

As the NSW Food Authority's Shellfish Program has evolved, the option for local harvest programs to utilise more advanced methods for chemical analysis has created an opportunity to compare quantified toxin levels and regulatory limits for phytoplankton cell concentrations. We examined sporadic blooms of *Pseudo-nitzchia* spp., *Alexandrium* spp. and *Dinophysis* spp., comparing Jellett Rapid test kits, high performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis and phytoplankton cell concentrations. Our data indicate that negative Jellett tests were consistent with detections of amnesic shellfish toxins below regulatory limits, as reported by LC-MS/MS. However, during an immense (>6 million cells/L) bloom of *Pseudo-nitzschia cuspidata* in 2010, we observed inconsistent results between chemical methods. We have found, for *Alexanandrium catenella* events, that Jellett paralytic shellfish toxin positives have been over-conservative when compared to HPLC analysis. *Dinophysis acuminata* events have also been complex. Cell concentrations above the regulatory limit (1,000 cells/L) do not always correspond with positive biotoxin reports in oysters, while there is an apparent seasonal trend in diarrhetic shellfish toxins in wild harvest stock (pipis). The continued use of chemical analytical methods will enable improved management of harvest programs and public health risks due to the apparent increase in algal-related toxic episodes.

Keywords: phytoplankton monitoring, shellfish harvest area management, algal biotoxins, risk assessment.

Introduction

Farm gate value of the New South Wales (NSW) shellfish industry is in excess of $AUD30 million annually (Creese and Trenaman, 2013). The native *Saccrostrea glomerata* is the principle species cultured, followed by *Crassostrea gigas* (diploid and triploid) in selected areas and *Ostrea angasai*. One harvest area produces *Mytilus edulis* (with two additional areas currently undergoing classification). Targeted species for wild harvest are pipis (*Donax deltoides*) and cockles (*Katelysia* spp.). Since 2005, management of NSW shellfish harvest areas has included regular phytoplankton and biotoxin monitoring of 74 harvest areas across 28 embayments and estuaries, over 2,000 Km of coastline (28° 10'-37° 30' S, Fig. 1). Management and monitoring of seasonal (June to November) wild harvest on up to 36 beaches has been regulated by the NSW Food Authority since 2005. Prior to 2012, Jellett Rapid test kits were the primary resource for screening algal toxins in shellfish tissue. While providing a fast and inexpensive screen for the three main toxin groups (amnesic shellfish toxins (ASTs), paralytic shellfish toxins (PSTs) and diarrhetic shellfish toxins (DSTs)) found in NSW coastal waters, the inability of the test to provide a measure of toxin levels was restrictive for industry. Quantitative testing capabilities were limited. One NSW laboratory offered analytical testing for ASTs and quantification of PSTs was carried out via mouse bioassay in South Australia. The nearest facility that could offer a complete toxin analysis service was based in New Zealand. With the logistical difficulties of transporting time-sensitive samples to another country, this option was impractical for routine use by Australian industry members.

Extended harvest area closures due to consistent Jellett biotoxin positives have been occasional and sporadic. When possible, during these events, additional sampling for comparison of Jellett, HPLC or LCMS/MS and phytoplankton cell concentrations was carried out. During 2012, a tender for analytical shellfish biotoxin analyses was issued for a centralised laboratory, based in Sydney. The shift to quantitative analysis has improved risk management for harvest areas, as it

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is now possible to relate algal abundance to biotoxin concentrations.

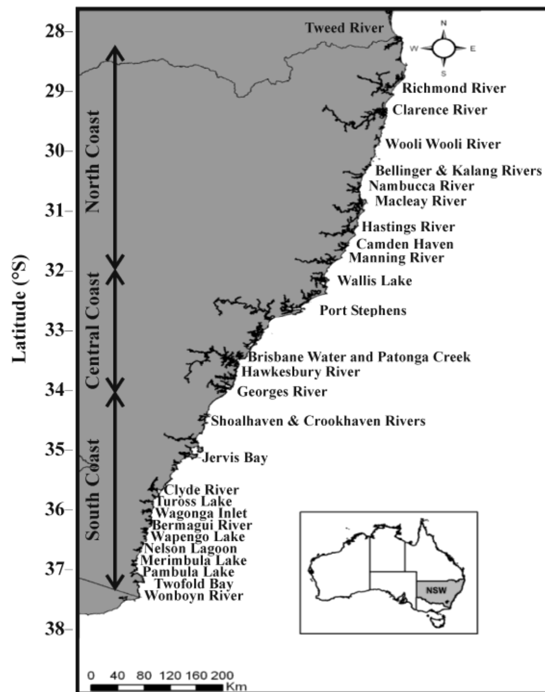

Fig. 1. Location of shellfish harvest areas, NSW coastline, Australia.

Material and Methods

All routine monitoring sites in each shellfish harvest area were selected to represent the water being filtered by farmed shellfish and in accordance with the criteria specified in the NSW Shellfish Program Marine Biotoxin Management Plan (MBMP) (NSW Food Authority (FA), 2014) and the Australian Shellfish Quality Assurance (ASQAP) Manual (2009). All samples were processed in accredited laboratories approved by the NSW Food Authority (2014).

Since 2005, more than 15,000 biotoxin tests for ASTs, DSTs and PSTs have been processed. Biotoxin content in shellfish was determined by either Jellett screening (PSTs, ASTs and DSTs, Jellett Rapid Testing Ltd, Canada) or by HPLC (AOAC 2005.05) or LCMS/MS (AOAC 991.26 van den Top et al., 2011, Villar-Gonzalez et al., 2011). Occasional samples for PSTs were quantified by mouse bioassay (MBA, AOAC 959.08) Concurrently, more than 12,000 water samples have been analysed for potentially harmful phytoplankton species. Briefly, phytoplankton cell counts from oyster harvest areas were carried out on 500-1,000 mL seawater samples, which had been concentrated by gravity-assisted membrane filtration. Seawater samples (50 L) from beaches where wild harvest occurred were concentrated by 20 µm mesh (500 mL) prior to analysis.

Results and Discussion

Our data showed harmful algal trends shifting between potential AST, PST and DST producers, over time and between estuaries. Summaries of the temporal and spatial distribution of potentially harmful species in all NSW shellfish harvest areas between 2005 and 2011 can be found in Ajani *et al.* (2013a) and Farrell *et al* (2013). This paper focuses on isolated bloom events where toxin content in shellfish was analysed by different analytical methods.

Alexandrium spp.

Alexandrium minutum, A. catenella Group IV ribotype (= *A. pacificum*) and possibly *A. tamarense* Group V (= *A. australiense*)) are the main source of PSTs in NSW coastal waters (Farrell *et al*., 2013). Occurrences of *Gymnodinium catenatum* are rare (Ajani *et al*., 2013a) and have not been linked to PSTs in NSW oyster harvest areas (Farrell *et al*., 2013, NSWFA unpublished data). To date, incidences of PSTs above the regulatory limit have not been recorded. During September/October 2009, Patonga Creek on the central coast (Fig. 1) was closed to shellfish harvest for five weeks due to positive PST (Jellett test kit) results. Although *Alexandrium* spp. cells were not detected in water samples from Patonga Creek at this time, *Alexandrium catenella* was detected (500 cells/L) in the neighbouring Hawkesbury River (Fig. 1).

Comparison of PST concentrations from HPLC, MBA and Jellett testing showed that negative Jellett tests occurred at or near the limit of detection of the other two test types (Table 1). Positive Jellett results coincided with quantified values at less than 50% of the regulatory limit (0.8 mg/Kg STX eq., Table 1). This suggested that Jellett testing was sufficiently sensitive for consumer protection but was limiting to industry given that the PST concentrations reported were below regulatory closure limits. To date, maximum levels of PSTs in NSW shellfish harvest areas have been 0.66 mg/Kg (~83% of regulatory limit) or less.

Table 1. Summary of HPLC, MBA and Jellett analysis of shellfish tissue samples from Patonga Creek and Hawkesbury River, October 2009.

		Patonga Creek (North & South)	Hawkesbury River	Patonga Creek (North & South)	Patonga Creek (south)	Patonga Creek (North)
Date		13/10/2009	17/10/2009	17/10/2009	22/10/2009	22/10/2009
PST STX eq.	mg/Kg	0.14	0.04	0.17	0.24	0.03
AOAC Mouse Bioassay	mg/Kg	0.34	<0.4	0.37	0.38	<0.4
Jellet PST Kit (NATA Extraction)	+/-	Positive	Negative	Positive	Positive	Negative

***Pseudo-nitzschia* spp.**

During 2010, at Wagonga Inlet on the south NSW coast (Fig. 1) an immense bloom of *Pseudo-nitzschia cuspidata* (>6 million cells/L, Fig. 2) caused severe economic losses up to $AUD1 million with harvest area closures lasting 16 weeks (Ajani *et al*., 2013b). This unusual bloom event occurred in the wake of an extreme bush fire through the catchment followed by severe flooding, which washed a significant amount of burnt material into the lake (A. Zammit *pers comm*). *P. cuspidata* dominated the total planktonic algal population (>93% during bloom maximum). During the event (Fig. 2), domoic acid (DA) concentrations were determined by HPLC (max 34 mg/Kg DA, Fig. 2). In general, Jellett testing was consistent with HPLC results. Jellett negatives coincided with ~25% of the regulatory limit (20 mg/Kg DA). One false negative Jellett result corresponded to 19mg/Kg DA. The food safety risk of this false negative was low, as the harvest area re-openings are linked not only to biotoxin results but also increased testing of algal concentrations (NSW FA, 2014), particularly following a bloom of this magnitude.

The increased routine use of LCMS/MS, coupled with the method's greater sensitivity, has seen increased reporting of ASTs. Between November 2012 and September 2014, 104 AST positives were reported. Prior to this, 38 Jellett AST positives since 2005 were reported. However, with the increased detections maximum concentrations were < 5 mg/Kg DA and the majority of samples (75%) were less than 1% of the regulatory limit (20 mg/Kg). In terms of consumer protection, these toxin levels have coincided with cell concentrations of *Pseudo-nitzschia* spp. above the BMP trigger levels (*P. delicatissima* group: 500,000 cells/L, non-*P. delicatissima* group: 50,000 cells/L). With continued data, there may be potential to increase testing triggers. However toxicity of *Pseudo-nitzschia* spp. can depend on life cycle stages, strain and environmental factors (Lelong *et al*., 2012) and further work is needed to elucidate toxic and non-toxic strains in NSW (e.g. DA was not detected in a strain of *P. cuspidata* from the northern NSW coast (Wallis Lake, Fig. 1) Ajani *et al*. (2013b)).

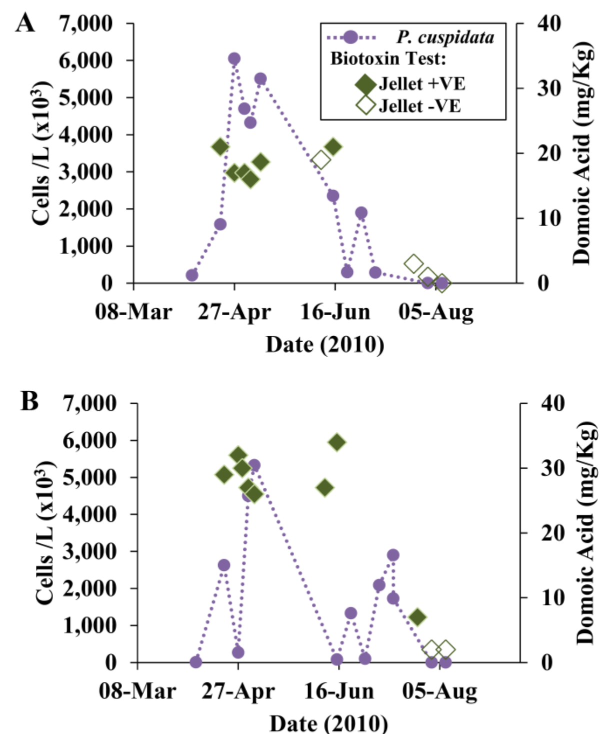

Fig. 2. Temporal distribution of *P. cuspidata* and DA in shellfish flesh at (A) western and (B) eastern harvest areas in Wagonga Inlet (Fig. 1) during 2010. Quantities of DA from HPLC analysis of oyster flesh and the corresponding positive/negative Jellett test result during the same period are provided.

***Dinophysis* spp.**

Cell proliferations of *Dinophysis* spp. in NSW harvest areas are primarily *Dinophysis acuminata* with some *Dinophysis caudata* (Ajani *et al*., 2013a). Annual detections of DSTs have been variable (Fig. 3), with occurrences primarily between July and March each year, with some incidence in June (Ajani *et al*., 2013a). Available data (2007-2008) in Richmond and Manning River harvest areas (Fig. 1) has found *Dinophysis*

acuminata up to 4,000 cells/L to be associated with low levels of DSTs (0.03-0.04 mg/Kg pectenotoxin2 seco acids and 0.03 mg/Kg dinophysistoxin 3 (DTX-3) detected in Sydney rock oysters. Contrastingly, concentrations of *D. acuminata* in pipi (wild) harvest areas of 2,500 cells/L during the same period yielded up to 0.44 mg/Kg DTX-3.

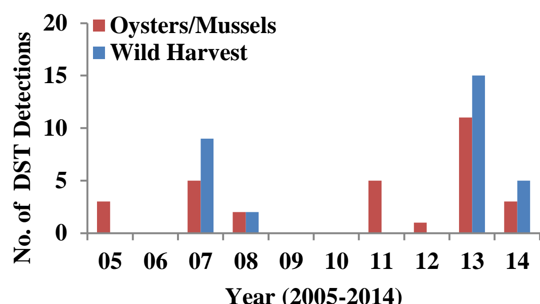

Fig. 3. Total number of DST detections in wild harvest shellfish and oysters/mussels between February 2005 and December 2014.

The above and more recent results (2013-2014) have demonstrated that pipis accumulate DSTs seasonally (August-December) at two-three times the regulatory limit (0.2 mg/Kg), when ~2,000 cells/L of *D. acuminata* were detected. In 2013, up to 0.4 mg/Kg Okadaic acid (OA) was detected in samples from Ballina region in northern NSW. During 2014, up to 0.58 mg/Kg OA was detected in samples from Stockton on the NSW central coast (Fig. 1). On both occasions, DSTs were detected for more than four weeks post the bloom peak.

In NSW shellfish harvest areas significant algal events have been infrequent. The majority of harvest area closures have been due to rainfall and/or salinity exceeding the trigger levels used as indicators of microbial and viral water quality. However, increasing demand on coastal resources from an increasing population (ABS, 2014) and the potential for spatial and temporal distributions of harmful algal species to be altered dramatically by a changing climate (Hallegraeff, 2010) are key future challenges. The application of quantitative methods in routine biotoxin risk assessment enables a better-informed approach for management of shellfish harvest programs, including a faster return to harvest on some occasions, and potential public health risks.

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Ichthyotoxic *Karlodinium* cf. *veneficum* in the Swan-Canning estuarine system (Western Australia): towards management through understanding

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Abstract

Blooms of the potentially ichthyotoxic dinoflagellate *Karlodinium* cf. *veneficum* have been associated with fish-kill events in the Swan-Canning estuarine system (Perth, Western Australia) since 2003. The greatest *K.* cf. *veneficum*-associated fish mortality was approximately 230,000 fish in 2003 while the most persistent bloom was a 100-day long event in 2012. These blooms occur on an almost annual basis, most commonly during the Austral autumn, and are associated with stratified conditions with benthic hypoxia brought about by the beginning of new freshwater flows. Such conditions can occur in late summer or mid-winter however at such times water temperature is rarely favourable for *K.* cf. *veneficum*. Bloom events are often terminated by significant weather events bringing stronger flushing and reduced water temperature. Fish-kills are often associated with a stress event to the bloom and cell lysis leading to release of karlotoxins into the water column. Understanding bloom dynamics and toxin profile are important for developing and applying effective control techniques. Histological investigation has found severe cytotoxic impact to the gill lamellae of impacted fish. The resident *K. veneficum* population has historically produced 6-oxo-KmTx2 karlotoxin however during the 2012 bloom a novel and potentially more toxic karlotoxin congener was detected.

Keywords: Climate change, endotoxin, hypoxia, karlotoxin, mixotrophy, stratification.

Introduction

Blooms of the small, toxigenic dinoflagellate *Karlodinium veneficum* have been recorded in the Swan-Canning estuarine system (Perth, Western Australia; Figure 1) since the late 1990's. While this identification has been confirmed via molecular techniques (Hallegraeff *et al*. 2010; Place *et al*. 2012), regular phytoplankton monitoring is via light-microscopy and confident identification to species is not always possible thus we identify as *K.* cf. *veneficum*.

The first fish-kill directly attributed to *K.* cf. *veneficum* in the Swan-Canning Estuarine System (SCES) was recorded in 2003 and this event remains the largest recorded fish-kill in the system, with 25,580 dead fish collected and an estimated mortality of approximately 275,000 fish. Fish-kills associated with *K.* cf. *veneficum* blooms have since been recorded in the Swan Estuary 2004-2006, 2010 and 2012 and in the adjoining Canning Estuary in 2003 and 2004. Isolates of *K. veneficum* from the SCES have previously been shown to produce KmTx2-1 (Adolf *et al*. 2005; Place *et al*. 2012) with a 2 h

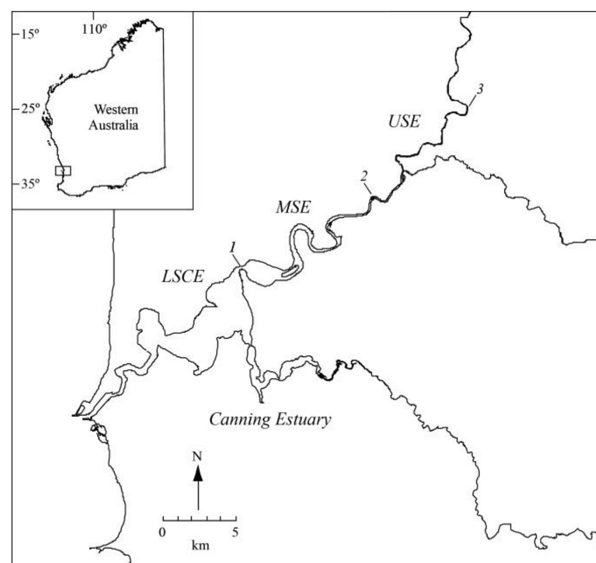

Fig. 3: The Swan Canning estuarine system. Data presented here are from monitoring in the Middle Swan Estuary (MSE) and Upper Swan Estuary (USE) between 1 (18 km upstream from the coast) and 3 (47.5 km upstream). 2 indicates the boundary between the MSE and the USE.

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LD_{50} of 563 ng/mL for sheepshead minnow (Mooney *et al*. 2010). While levels of this toxin have been recorded well in excess of the LD_{50} and characteristic gill damage observed (severe epidermal hyperplasia of the secondary lamellae), low levels of dissolved oxygen are also thought to be a contributing factor.

K. cf. *veneficum* HABs are now a major concern for management authorities. Understanding bloom dynamics is important for guiding both long-term catchment management and potential short-term in-river bloom mitigation stratagies.

Here we present 7 years worth of Swan Estuary monitoring data between 2003 and 2012 with a focus on *K.* cf. *veneficum*. This data illustrates some of the environmental controls on *K.* cf. *veneficum* and presents the detection of a novel putative karlotoxin.

Material and Methods

Environmental monitoring of the Swan Estuary from harbour entrance to over 50 km upstream is conducted on a weekly basis. Extra sampling sites are included and sampling occurs more frequently when a HAB is detected. Physicochemical and biological parameters are collected via discrete water samples (nutients, TSS, pigments) and sonde depth profiles (temperature, conductivity, salinity, dissolved oxygen, turbidity, chl-fluorescence and pH). Depth-integrated phytoplankton samples are collected with 50 mm diameter hose-pipe and immediately preserved with lugol's iodine solution and enumeration and identification performed via light-microscopy. All data used here was downloaded from the Water Information Reporting (WIR) database (www.water.wa.gov.au) or received directly from WIR officers at the Department of Water, Western Australia.

During the 2012 *K.* cf. *veneficum* bloom event samples were collected for karlotoxin analysis. This was done at response sites when depth-integrated density of *K.* cf. *veneficum* was $>3.0\times10^4$ cells/mL. On each occasion a syringe was used to force 2 – 5 mL of non-preserved phytoplankton sample water though a 13 mm hydrophilic PTFE acrodisc filter with 0.45 μm pore size. characterization of toxins and their concentrations was performed via HPLC and reverse phase LC-MS following the methods of Bachvaroff *et al*. (2008).

Results and Discussion

K. cf. *veneficum* most commonly occurred in bloom densities in the Austral autumn, though significant blooms have impacted the system during other seasons. Perth experiences a mediterranean-type temperate climate with hot, dry summers and cool, wet winters. Rainfall associated with the remnants of cyclones at lower latitudes commonly breaks the summer drought in February while temperatures remain high. Due to the dry catchment any flow produced by these events is generally minimal but freshwater input from local drainage can be sufficient to result in a halocline in the narrower mid- and upper-reaches of the estuary. As well as bringing nutrients off the catchment these conditions promote hypoxia in the deeper waters and nutrient release from sediments. Such conditions appear to promote formation of *K.* cf. *veneficum* blooms however while blooms were initiated in March during 4 of the 7 bloom years analysed, only low intensity blooms that do not persist were recorded during February. *K. veneficum* (Swan River isolate KVSR01) is essentially euryhaline but growth rate has been shown to rapidly decline from a peak at 20°C to negative growth at 25°C (Hallegraeff *et al.* 2010). Our hypothesis is that water temperatures between 25 and 30°C during the summer and early autumn period limit the ability of *K.* cf. *veneficum* to exploit the otherwise favourable conditions. Water temperatures drop below 25°C by mid-March and blooms generally occur from this period should moderate and sporadic rainfall events promote freshwater input and stratification characteristic of a salt-wedge estuary without creating significant flow (Figure 2). As indicated for the Neuse River Estuary (Hall *et al*. 2008), small-scale hydrological forcing and vertical migration can result in cell concentrations peaking between 1.0×10^5 – 2.0×10^5 cells/mL.

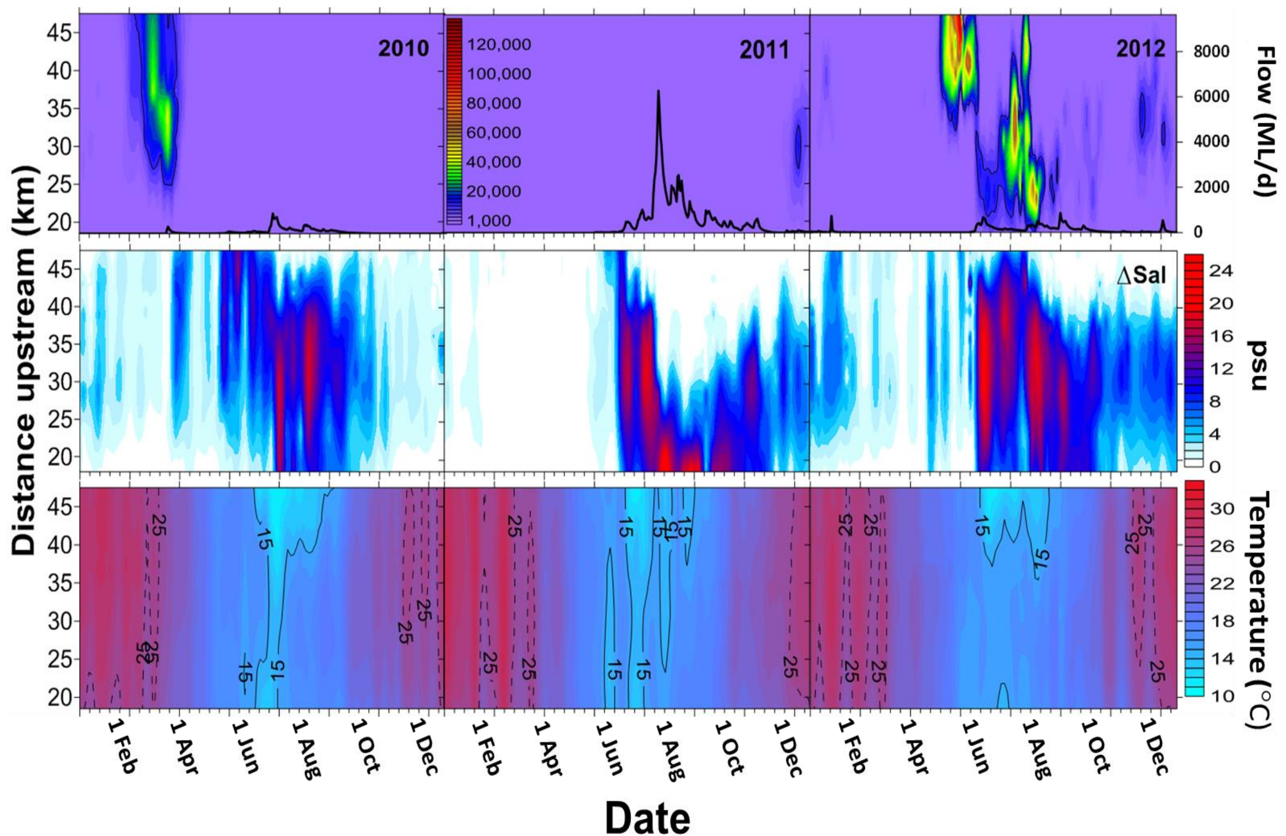

Fig. 2: (top) *Karlodinium* cf. *veneficum* density contours for 2010-2012 in the middle to upper reaches of the Swan Estuary and total inflow (black line) recorded from the Avon River and Ellen Brook which provide the vast majority of catchment-fed flow to the estuary; (middle) Delta-salinity as a proxy for level of stratification; and (bottom) water temperature at a depth of 1.5 m.

The demise of autumn *K.* cf. *veneficum* blooms is often brought about by the onset of persistent winter rains that promote greater flow, reduced salinity and water temperatures <17□C. Examples of this were seen in the data from 2004-2006 and 2010 (Figure 2). In 2011 the summer and autmn months were extremely hot and dry and the sudden onset of winter weather in June left no growth window for *K.* cf. *veneficum*. In contrast to this, lack of significant inflow, persistent stratification and higher than normal winter water temperates appear to be the reason for an unusual *K.* cf. *veneficum* bloom that persisted throughout winter 2012 (Figure 2).

Sampling for karlotoxins (May-August) during the extended bloom sequence in 2012 detected toxins associated with high densities of *K.* cf. *veneficum* (Figure 3). Over ten days prior to a fish-kill in the upper Swan Estuary on day 153 (1 June), elevated levels of 6-oxo KmTx2-1 had been detected (peak 80.35 ng/mL; Figure 3).

Fish sampled in the evening of day 152 were found to have significant gill damage (pers. comm. Jo Bannister) but were actively feeding (personal observation, S. Hoeksema).

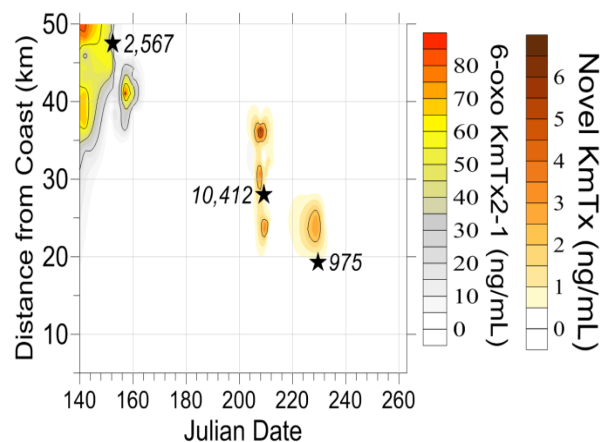

Fig. 3: Karlotoxin concentrations measured in the middle and upper Swan Estuary during *Karlodinium* response sampling in 2012. Stars indicate fish kills. Values correspond to the number of dead fish collected on each occasion.

Heavy rainfall in the late evening resulted in intense stratification and although daytime monitoring did not detect hypoxia a logger further downstream recorded nighttime surface dissolved oxygen concentrations <2 mg/L. The first dead fish were reported the following day and it is

hypothesized that with oxgen exchange already compromised the fish would have quickly succumbed to hypoxia and been less capable of moving away from hypoxic zones.

The rainfall event at the end of May 2012 resulted in significant river flow and the bloom was forced almost 20 km downstream (Figure 2). Over the following 2 months the weather was unseasonally warm and the bloom reformed and progressed upstream with tidal forcing. Measured levels of a novel, uncharacterised karlotoxin peaked at 8.1 ng/mL on 26 July (day 208) and a major fish kill was reported the following day (Figure 3). Another smaller fish kill further downstream 20 days later was also associated with low levels of this uncharacterised karlotoxin. This compound had two later eluting peaks; major, m/z 1349.9 (sodiated, so MW would be 1326) and minor m/z 1329.7 (sodiated, so MW would be 1306) that distinguishes it from other known karlotoxin congeners. This final fish kill did not appear to be co-occurring with hypoxic conditions and the timing and location of both of these events provides some evidence that this new toxin may be a more toxic KmTx congener.

Future work will focus on characterisation of the novel karlotoxin via large-scale filtration and solid phase extraction (Rundberget *et al.* 2007) and pryrosequencing of bloom populations to better characterise the population through a bloom sequence.

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Micro-phytoplankton and harmful species distribution and abundance in fjords of Southern Chile

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Abstract

Harmful species of *Alexandrium*, *Dinophysis*, *Protoceratium* and *Pseudo-nitzschia,* are present in Chilean fjords sometimes in bloom conditions or associated to toxic outbreaks. Data from 2006 to 2013 were analysed to understand the composition, abundance and distribution of the micro-phytoplankton, including harmful species, in the Reloncaví (RF, 42°40'S) and Puyuhuapi fjords (PF, 44°40'S) and Union channel (UC, 52°00'S). Diatoms contributed not less 98% of total cell density, with *Skeletonema spp.*, contributing from North to South with 90, 39 and 28% of total density. The harmful species were numerically subordinate (<1% of total number), with the exception of *Pseudo-nitzschia* cf. *pseudodelicatissima* and *P.* cf. *australis* that both contributed 45% of total density in PF. Both species were present in low numbers in RF (0.3%) and UC (5.5%). *Alexandrium catenella*, *Alexandrium ostenfeldii* and *Dinophysis acuta* were absent in RF; these species plus *Dinophysis acuminata* and *Protoceratium reticulatum* were present in the other two sectors. The studied areas span climatic and oceanographic differences due to their latitudinal position and local land-sea interactions (e.g. river inputs and glacier melting), that determine differences in composition and density of total micro-phytoplankton, including dissimilarities in composition and abundance of harmful species.

Key words: Micro-phytoplankton, harmful species, fjords, Southern Chile

Introduction

The fjords of Southern Chile exhibit a high hydrographic variability, modulating the structure and the organization of the planktonic communities. In the last four decades recurrent harmful algae blooms have been observed, mainly caused by *A. catenella*, associated to PSP outbreaks (Guzmán *et al*., 2002; Varela *et al*., 2012; Díaz *et al*., 2014), seriously affecting public health and the coastal economy (Suárez-Isla *et al*., 2002). Therefore, the goal of this study was to describe across a broad latitudinal area, the distribution, composition and abundance of the micro-phytoplankton assemblages associated with harmful and potentially harmful species.

Material and Methods

The Reloncaví (RF, 42°40'S) and Puyuhuapi (PF, 44°40'S) fjords and Union channel (UC, 52°00'S) were sampled monthly from May 2006 to February 2013 (Fig. 1). For each of 16 sampling sites (Fig.1), water samples from surface to 10m depth, were collected. Data analysis considered phytoplankton composition and numerical abundance using Utermöhl method (1958) both at the species level. Temperature and salinity data from 5m depth were also documented.

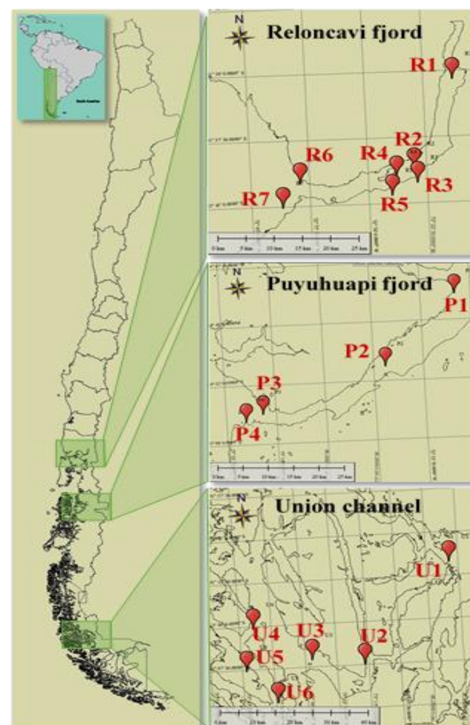

Fig.1. Sampling sites in the studied sectors.

Data analysis included information from each annual period, and data split into Spring-Summer and Autumn-Winter. Each period comprised 4 to

6 samples per sampling site. From numerical abundances the mean (±SE) of species richness (Margalef), Shannon-Wiener diversity index (*H*'), Evenness (*J*') and G index (Clément & Guzmán, 1988), were estimated. Cumulative abundance (cells L^{-1}) was estimated adding the total phytoplankton abundance per sampling site in each studied sector.

Results

The diatoms were the dominant taxa contributing not less than 98% of total micro-phytoplankton abundance. Independently of this dominance, the data showed spatial and temporal differences. The cumulative abundance was higher ($p<0.05$) at RF compared to the other two sectors, reflecting a decreasing gradient from North to South in both seasonal periods (Fig. 2). This was mainly explained by the contribution of *Skeletonema* spp. (90, 39 and 28% of total cumulative abundance). Well-marked temporal variations in micro-phytoplankton densities were observed (Fig. 2). The lowest (mean±SE) cumulative density ($1.5\times10^5 \pm 6.9\times10^4$ cells L^{-1}) was observed during Autumn-Winter at UC, whereas the highest density ($2.5\times10^6 \pm 1.9\times10^5$ cells L^{-1}) was observed at RF during Spring-Summer. This was due to *Skeletonema spp*., when higher temperature (14.2±0.9°C) and a low salinity was observed (22.3±2.5psu). This dominance decreased for PF and UC sectors, where *P.* cf. *pseudodelicatissima Chaetoceros socialis* and *C. debilis* were numerically important (Fig.2).

Cumulative abundance and species numbers showed opposite gradients at RF and PF, while UC did not show any tendency. The cell density decreased from the fjord head (1.2×10^8 and 3.3×10^7) to the fjord mouth (4.7×10^7 and 2.8×10^7) at RF and PF, respectively. In contrast, the species number increased towards the fjord mouths from 86 to 147 and from 94 to 135 species at RF and PF, respectively (Fig. 3).

The highest species number was observed in the region of Aysén (PF). The number of species identified at RF, PF and UC comprised 92, 142 and 89 species during Spring-Summer and 69, 111 and 53 species during Autumn-Winter, respectively. Although seasonal estimations of the species richness (Margalef index) were similar in each studied sector (Fig.3; ANOVA, $p>0.05$), significant differences between sectors were observed (ANOVA, $p<0.001$), being higher at PF than the other two sectors (Scheffé test, $p<0.001$).

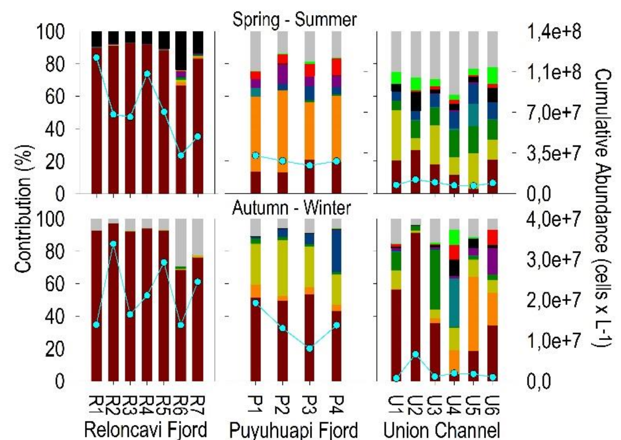

Fig.2. Seasonal total cumulative abundance (cyan dots) and species contribution to total abundance (bars) for the three sampled sites.

Although seasonal estimations of the species richness (Margalef index) were similar in each studied sector (Fig.3; ANOVA, $p>0.05$), significant differences between sectors were observed (ANOVA, $p<0.001$), being higher at PF than the other two sectors (Scheffé test, $p<0.001$). The Shannon-Wiener index showed low values in all sampling sites ($H'<1.6$) with similar seasonal estimations (ANOVA, $p>0.05$), being significantly different between sectors (ANOVA, $p<0.001$), and RF presented the lowest estimates (Scheffé test, $p<0.001$) reflecting the numerical dominance by few species during the study period (i.e. *Skeletonema spp*; Fig. 3). The Evenness index estimations (*J*', Fig. 3) showed a significant increase with latitude (ANOVA, $p<0.001$) reflecting a more homogeneous numerical abundance between species toward the South; but no seasonal differences between sectors were observed (ANOVA, $p>0.05$). Finally, the G index (>0) for all sampling sites (Fig. 3) revealed the dominance of diatoms; with significant differences between sectors (ANOVA, $p<0.001$) and the lowest estimates (Scheffé, $p<0.001$); and seasonal differences (ANOVA, $p<0.001$) at PF.

Seven toxic or potentially toxic species were identified (Fig. 4). The most abundant species were the diatoms *P.* cf. *pseudodelicatissima* and *P.* cf. *australis*, which showed a wide geographic distribution and were present at the three sampled sectors; the highest total abundance was registered at PF during Spring-Summer (>50%).

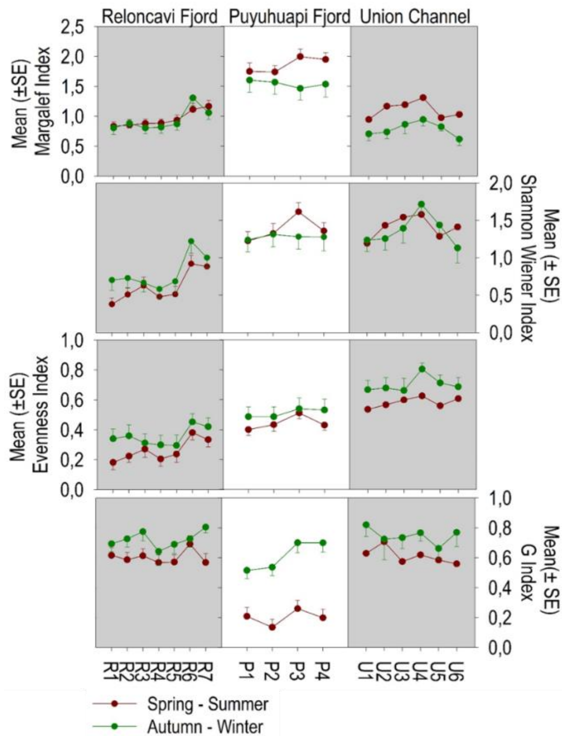

Fig. 3. Variation in ecological indices for each site and sampled sector.

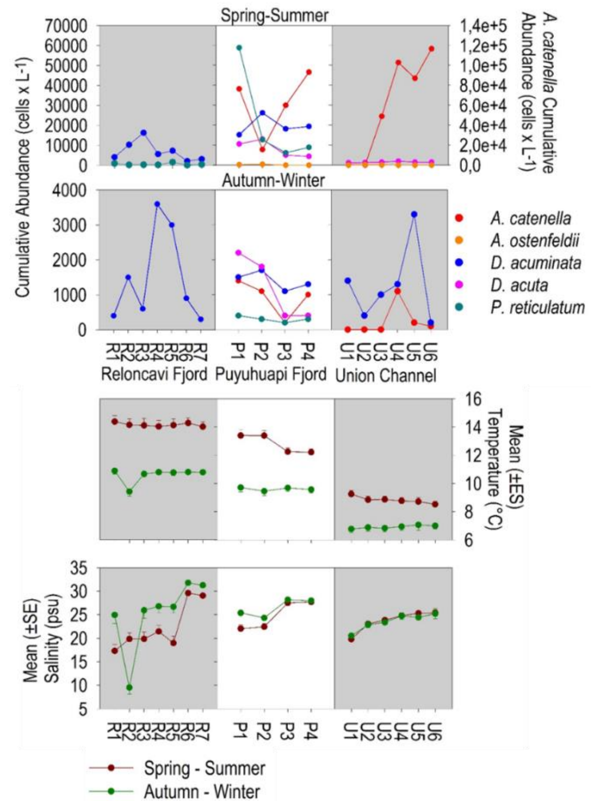

Fig.4. Variation of cumulative abundance of harmful dinoflagellates, temperature and salinity.

While, the toxic dinoflagellates species showed a low contribution to total cumulative abundance (<1.0%). *A. catenella* was absent from RF, but numerically relevant, in UC during the Spring-Summer period (<0.7% of total abundance), whereas, *A. ostenfeldii* was present at PF and UC, but with low densities (600 cells L^{-1}; 0.001% total abundance). *D. acuminata* showed the widest geographic distribution and low densities (<0.1% of total abundance) occurred in the three studied sectors. *D. acuta* and *P. reticulatum* were absent from RF and UC, respectively. Seawater temperature (5m depth) ranged from 6.8ºC at UC during Autumn-Winter to 14.4ºC at RF during Spring-Summer. In general, a latitudinal temperature decrease and marked seasonal differences (ANOVA, p<0.001) were observed; within each sector a temperature gradient from East to West in Spring-Summer is observed associated to the more eastern sampling stations (Fig.4). For each studied sector an East to West gradient was observed, with the lowest values recorded in the eastern stations.

Excluding values recorded at UC and at the head of PF (P1 and P2), the salinities showed significant differences between seasonal periods (ANOVA, p<0.001). These were most marked at RF with the lowest salinities during Spring-Summer (Fig. 4).

Discussion

Similarly to other studies conducted in Chilean fjords (e.g. Clement & Lembeye, 1993; Alves-de-Souza *et al.*, 2008; Montecino *et al.*, 2009), a clear dominance of diatoms (in terms of species number and their abundance) in micro-phytoplankton assemblages was encountered. Harmful dinoflagellates species represented a very minor fraction of the total abundance. The study sites, covered a wide geographic area, with different geomorphologies and differences in the land-sea interactions (e.g. water run-off, river discharges and glacier melting) (Bastén & Clément, 2005; Sievers, 2006; Schneider *et al.*, 2014). These traits modulate the composition, distribution and abundance of micro-phytoplankton assemblages (Paredes *et al.*, 2014), which are reflected by the biological indices utilized and the correspondence with environmental variables as seawater temperature and salinity. These conditions may

determine the composition and abundance of certain species, such as *Skeletonema spp*. that showed the highest abundances and a wide geographic distribution, features that have been previously pointed out (Avaria *et al*., 2004; Valenzuela & Avaria, 2009). However, local conditions may drive the *Skeletonema spp.* dominance, favouring the abundance increase of other diatoms species (i.e. *Pseudo-nitzschia spp*., *C. debilis* and *C. socialis*). Among the harmful dinoflagellates, *A. catenella* was quantitatively the most important species; although it was absent from RF, in contrast to a previous study that cited this species in this area (Valenzuela & Avaria 2009). Besides, it has been pointed out the presence of PSP suggesting *A. catenella* as the primary source (Seguel *et al*., 2006), although it cannot be rule out *A. ostenfeldii* as the source of this toxic complex. The seawater temperature and salinity was associated with *A. catenella* abundance. This species was absent from low salinity sites (<23.5 psu) and higher abundances occurred during Spring-Summer when the temperature was higher. Vidal *et al.* (2012) have shown for Chilean fjords a relationship between *A. catenella* abundance and seawater temperature. The results showed that *D. acuminata* was present in all the studied sectors, coinciding with the wide geographical distribution found previously (Muñoz *et al*., 1992; Cassis *et al*., 2002). This distribution is explained by the broad tolerance to temperature and salinity shown by this species (Díaz *et al*., 2011), and the effect of water column stability and nutrient availability (Margalef, 1978).

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Alexandrium and risk management within the Scottish phytoplankton monitoring programme

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Abstract

The potentially harmful genus *Alexandrium* is frequently detected in Scottish coastal waters during routine regulatory monitoring of shellfish harvesting areas. *Alexandrium* can be highly toxic and dense blooms are not required before there is cause for concern. Until July 2014 the phytoplankton trigger level to instigate additional flesh biotoxin testing was set at 20 cells L^{-1}, but in an effort to reduce the burden on aquaculture businesses, recent guidance issued to harvesters advised that classified shellfish production areas be moved to a higher alert status only if *Alexandrium* was present in seawater at a concentration of 40 cells L^{-1} or greater, or had been at this level in the preceding two weeks. Paralytic shellfish toxin events were examined over a five year period between 2009 and 2013 to determine whether the application of the revised trigger level would have resulted in potentially harmful toxic events being missed. The analysis suggests that it is unlikely that toxins would have exceeded the regulatory limit before action was taken, and hence that the revised trigger level of 40 cells L^{-1} is unlikely to compromise food safety. The investigation highlighted the importance of weekly phytoplankton monitoring over the high risk period, as blooms can develop rapidly.

Keywords: Alexandrium, monitoring, shellfish safety, Scotland

Introduction

Shellfish production has great importance in terms of employment in the coastal communities of remote rural areas, with the Scottish Government having a target of effectively doubling production by 2020. In 2013 the industry had an estimated value worth £8.9 million, with common or blue mussels (*Mytilus edulis*) and Pacific oysters (*Crassostrea gigas*) contributing the greatest proportion in terms of both value and tonnage (Munro and Wallace 2014). To comply with EU legislation, official control monitoring of Scottish classified shellfish harvesting areas is undertaken by several UK laboratories, on behalf of the Food Standards Agency in Scotland (FSAS). Since 2012, the programme has been delivered through a consortium of these laboratories. Monitoring is based on a 'Pod' system, usually comprised of a number of production areas. Each Pod contains a Representative Monitoring Point (RMP), with common mussels frequently used as an indicator species. Any biotoxin result above the regulatory limit at the RMP will close all associated harvesting areas (AHAs) within that Pod. Harvesters in the AHAs may challenge the closure if end-product testing indicates that the shellfish, which may be a different species to those at the RMP site, are safe for consumption. Shellfish and seawater samples are collected from the RMPs by official control sampling officers at frequencies varying from weekly to monthly, determined by a risk assessment for each Pod. Increased testing takes place where phytoplankton levels indicate a greater risk. As part of the consortium, Cefas laboratory carry out testing on shellfish for toxins associated with diarrhetic, amnesic and paralytic shellfish poisoning. Phytoplankton monitoring is undertaken by SAMS in fewer Pods, although these are chosen on the basis of providing good geographic coverage, as well as targeting areas with high levels of harvesting activity, and those with historic toxicity. In 2013 there were approximately 80 active Pods covering about 275 sites, 40 of which were routinely monitored for phytoplankton (Stubbs *et al.* 2014).

The potentially harmful genus *Alexandrium* is frequently detected around Scotland (Fig. 1), and highly toxic Group I *Alexandrium tamarense* is known to be present in Scottish waters (Davidson and Bresnan 2009). In June 2014, a bloom of 7,280 cells L^{-1} recorded in Loch Striven (Argyll & Bute) resulted in Paralytic Shellfish Poisoning (PSP) toxins being detected in common mussel flesh at more than 18 times the regulatory limit of

A. Lincoln MacKenzie [Ed]. *Marine and Freshwater Harmful Algae 2014*. Proceedings of the 16th International Conference on Harmful. Algae. Cawthron Institute, Nelson, New Zealand and the International Society for the Study of Harmful Algae (ISSHA)

800 µg STX eq. kg^{-1}. Historic evidence indicates that regional 'hotspots' identified around Orkney, the Shetland Islands and the east coast of Scotland may be related to the presence of cyst beds (Bresnan *et al.* 2008). Regulatory monitoring since 2006 suggests that some west coast sea lochs may also fit this description, with PSP toxins frequently being reported, particularly in spring and early summer.

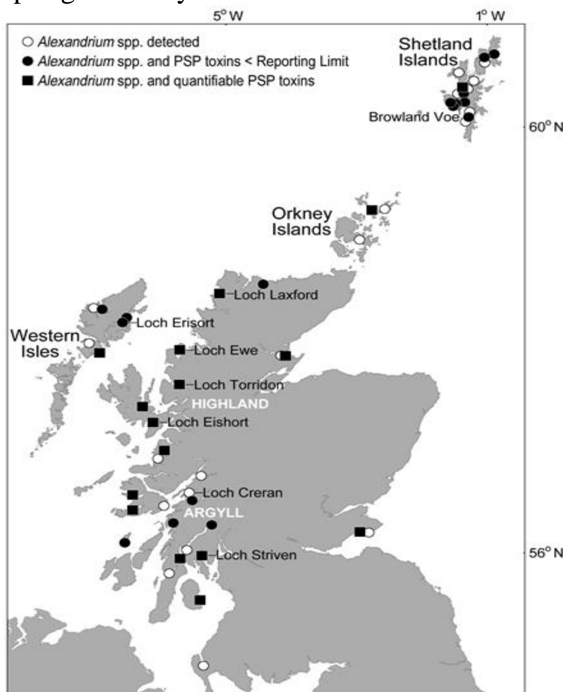

Fig. 1. Location of phytoplankton monitoring sites around Scotland where *Alexandrium* was recorded between 2009 and 2013. The relationship between *Alexandrium* and PSP toxins detected in shellfish tissue is shown.

PSP toxins often exceed the permitted level despite relatively low densities of *Alexandrium*. For example, *Alexandrium* cell counts in Loch Eishort (Highland) in 2013 were recorded at 80, 40 and 120 cells L^{-1} in the three weeks preceeding a PSP toxic event (Fig. 2). During this period, toxins were detected at levels below the reporting limit (RL), and did not exceed the regulatory limit until the following week, which coincided with an *Alexandrium* bloom of 180 cells L^{-1}. Shellfish were not tested for PSP between 01-July and 16-September due to closure of this site for diahrretic shellfish poisoning (DSP) toxins, despite *Alexandrium* reaching a density of 4,740 cells L^{-1} on 08-July. However, three other sites within the Highland region (Loch Torridon, Loch Ewe and Loch Laxford) showed similar peaks in *Alexandrium* density two to three weeks earlier than that recorded at Loch Eishort, with PSP toxins reported at above the regulatory limit for a period of five weeks at both Loch Torridon and Loch Laxford.

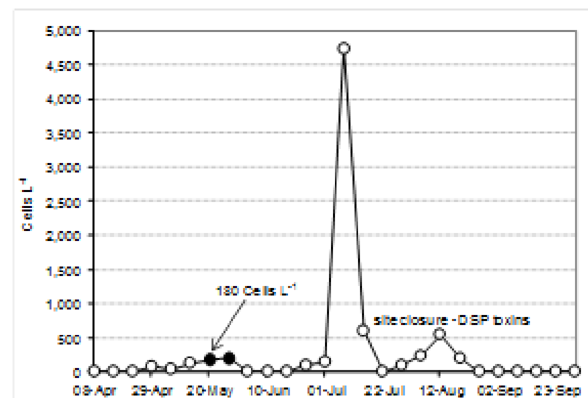

Fig. 2. *Alexandrium* counts (cells L^{-1}) by week for Loch Eishort in 2013. In PSP 'hotspots', toxins may exceed the regulatory limit (indicated by ●) when *Alexandrium* is present at relatively low densities.

Alexandrium blooms in Scottish waters have rarely been recorded at densities greater than 10,000 cells L^{-1} within the monitoring programme, although an exceptional bloom exceeding 61,000 cells L^{-1} was observed around the Shetland Islands in July 2009 (Table 1).

Table 1. Maximum cell densities by year for the genus *Alexandrium*, recorded as part of the monitoring programme between 2009 and 2013. ND = Not Detected.

Month Year	Location	Max. density cells L^{-1}	PSP toxicity
July 2009	Browland Voe	61,720	ND
May 2010	Loch Creran	3,100	ND
May 2011	Loch Creran	9,680	ND
July 2012	Loch Erisort	6,500	ND
June 2013	Loch Torridon	9,700	> reg. limit

Until July 2014, the *Alexandrium* trigger level to instigate additional flesh testing in shellfish was set at 20 cells L^{-1} i.e.'presence' in a 50 ml sub-sample of seawater. In an effort to reduce the burden on aquaculture businesses, some of which are relatively small enterprises, recent guidance issued to harvesters by FSAS (Anon. 2014) advised that classified shellfish growing areas be moved to a higher alert status only if *Alexandrium* was detected at a concentration of 40 cells L^{-1} or greater, or had been at or above this density in the preceding two weeks. Other considerations, such as toxicity in neighbouring areas and historic PSP

events at individual sites should also influence the decision of the harvester whether or not to increase end-product testing. The aim of this investigation was to determine whether the application of a revised *Alexandrium* trigger level of 40 cells L^{-1} was an effective approach to managing the risk of a PSP toxic event, through the analysis of a historic data set obtained as part of the official control monitoring programme.

Material and Methods

Quantifiable PSP toxic events in common mussels were examined between 2009 and 2013, when toxin results had been routinely obtained using instrumental methods, and closer integration of the SAMS and Cefas components of the monitoring programme increased the amount of phytoplankton and biotoxin data available for individual sites. The *Alexandrium* trigger level used to instigate additional flesh testing during this period was set at 20 cells L^{-1}. Seawater samples for phytoplankton analysis were obtained on a weekly basis between April and September, with reduced sampling frequency at other times. However, in 2013 the weekly sampling period was extended to include March. A total of 4,949 seawater samples were preserved in acidic Lugol's iodine solution to obtain a final concentration of 1% by volume, and processed using the Utermöhl sedimentation method (in Edler and Elbrächter 2010). Potentially toxic genera/species were enumerated in a 50 ml sub-sample by inverted light microscopy.

Results and Discussion

The percentage of *Alexandrium* detected at densities equal to or greater than 20 cells L^{-1} is shown in Fig. 3. *Alexandrium* was observed in 1,653 samples (33.4%) and was present at 20 cells L^{-1} in 9.6% of the samples analysed, a cell concentration that would no longer automatically lead to increased end-product testing for PSP toxins. A total of 672 bloom events were identified, in which *Alexandrium* was continuously detected (i.e. every week) at a monitoring site over a period lasting between one week and several months. It was recorded at densities not exceeding 20 cells L^{-1} in 210 (31%) of these events.

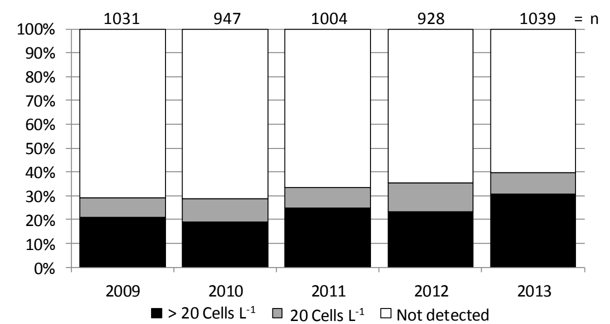

Fig. 3. Percentage of seawater samples in which *Alexandrium* was detected at densities equal to or greater than 20 cells L^{-1} between 2009 and 2013, where n = total number of samples per year.

Shellfish are not routinely tested for PSP as part of the official control monitoring programme if a site is already closed due to the presence of other toxins above the regulatory limit. For example, widespread closures lasting about three months occurred throughout the Shetland Islands in 2013, as a result of an extensive *Dinophysis* bloom and associated diahrretic shellfish toxins (Whyte *et al.* 2014). Data for PSP toxins, detected either at the start of a bloom or in the following two weeks, were therefore available for 358 (53.3%) bloom events between 2009 to 2013. Where testing occurred, PSP toxins were detected on 98 occasions (27%) with quantifiable results recorded for a third of these cases. Approximately 3% of all *Alexandrium* blooms tested had PSP toxins above the regulatory limit. In the majority of cases (70%), *Alexandrium* was observed in the water column at concentrations equal to or greater than 40 cells L^{-1} prior to PSP toxins being detected, thus highlighting the importance of weekly phytoplankton monitoring over the high risk period. *Alexandrium* was coincident with the onset of a PSP toxic event in a further 15% of cases. For the remaining events, it is possible that the start of the toxic event may have been missed on two occasions when *Alexandrium* densities were less than 40 cells L^{-1}, if there had been no consequent toxin testing. However, where data are available, *Alexandrium* levels had increased to greater than 40 cells L^{-1} before toxin concentrations exceeded 800 µg STX eq. kg^{-1}. The analysis therefore suggests that if the *Alexandrium* trigger level had been set at 40 cells L^{-1} between 2009 and 2013, it is unlikely that PSP toxins would have exceeded the regulatory limit before action was taken. Although a close link between *Alexandrium* and PSP toxins can be frequently demonstrated, it has often been the case that *Alexandrium* blooms do not correspond to

elevated shellfish toxicity. *Alexandrium tamarense* (North American Type I strain), *Alexandrium minutum* and *Alexandrium ostenfeldii* are all known toxin producers found in Scottish coastal waters (Brown *et al.* 2010), and frequently co-occur in the water column. However, non-toxic *Alexandrium tamutum* and *Alexandrium tamarense* (Western European Group III strain) have also been identified (Touzet *et al.* 2010) and some of the densest blooms recorded within the monitoring programme have not been associated with any shellfish toxicity (Table 1). The greatest abundance of *Alexandrium* recorded over the five-year period between 2009 and 2013 was observed in Browland Voe (Shetland Islands) in July 2009. PSP toxins were not detected at this time, although some toxicity had been detected earlier at this site in April 2009, associated with *Alexandrium* at a density of 120 cells L^{-1}. Similar patterns were observed at other sites in the Shetland Islands during 2009, with low density toxic *Alexandrium* present in spring and denser, non-toxic blooms occuring during the summer. In both 2010 and 2011, the densest *Alexandrium* blooms around the Scottish coast were both recorded in Loch Creran (Argyll & Bute) and *Alexandrium* was present continously for eleven weeks from March onwards in both years. Although several species of *Alexandrium* were observed, non-toxic *Alexandrium tamutum* appeared to dominate.

As it is not possible to routinely identify *Alexandrium* to species/strain level using light microscopy within the monitoring programme, a precautionary approach is taken by FSAS, and harvesters are advised to assume that the genus *Alexandrium* has the potential to cause a toxic event and thus respond appropriately with either an increased level of end-product testing, or voluntary suspension of harvesting. Elevated phytoplankton levels may also be used by FSAS to advise local authorities to close wild shellfish harvesting areas where sufficient toxin testing has not been undertaken. In conclusion, advice to harvesters that classified shellfish growing areas should be moved to a higher alert status if *Alexandrium* is detected in seawater at a concentration of 40 cells L^{-1} or greater would seem a robust approach to the risk management of PSP toxicity in shellfish.

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First confirmed report of fish-killing *Pseudochattonella* species (Dictyochophyceae) on the west coast of Canada

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Abstract

In late September 2013 a bloom of *Pseudochattonella* species caused mortality in farmed Atlantic salmon in an aquaculture facility in Quatsino Sound, British Columbia. The bloom was preceded by a storm event with high winds and heavy rain. *Pseudochattonella* cells detected early in the bloom were small round flagellated swimming cells, which developed into larger oval and oblong cells within a few hours. Mortality was observed at this time, and although the bloom persisted for several days it appears ichthyotoxicity was only seen during this period. Extreme pathology of the gill tissue was observed in mortalities, with gills appearing ragged, frayed and mucus-y. A culture was established from live samples obtained from the bloom. Real-time PCR analysis was performed using a published Taqman assay, and positive results confirmed the identification of *Pseudochattonella* species in the isolated culture. Although *Pseudochattonella* has been implicated in mortalities of farmed salmon in this area since 2007, identification to present has been based solely on cell morphology; this is the first molecular confirmation of that identification.

Keywords: harmful algae, ichthyotoxicity, *Pseudochattonella*, Atlantic salmon, real-time PCR

Introduction

Farmed salmon is the largest agricultural export in British Columbia (BC), with an annual production of ca. 70,000 tonnes, and a market value of ~CAD$500 million (Statistics Canada 2011). There are approximately 130 salmon farm tenures in BC, with 75-80 farm sites in operation at one time (Ministry of Agriculture 2011). Most farm sites are situated around Vancouver Island, although there are a few in the Central Coast area of BC.

Since its inception in the 1970's, salmon aquaculture in BC has been impacted by harmful algae species (Black 1990; Whyte *et al.* 1999; Taylor & Harrison 2002). In 1999 the salmon farmers started the Harmful Algae Monitoring Program (HAMP) with Fisheries and Oceans Canada (DFO) to monitor, manage and mitigate harmful algae blooms (HABs) in BC. Early days in BC aquaculture identified *Heterosigma akashiwo* and mechanically harmful *Chaetoceros* species as the most common causative species of fish kills; since 1999 there have also been farmed salmon kills due to *Dictyocha*, *Chrysochromulina*, *Chattonella,* and *Pseudochattonella* species (Haigh, unpubl. data).

Pseudochattonella species are a small group of ichthyotoxic microalgae known to affect fish in disparate areas of the globe, including Japan, Europe, and New Zealand (Yamaguchi *et al.* 1997; Hosoi-Tanabe *et al.* 2007; Backe-Hansen *et al.* 2001; McKenzie *et al.* 2011). Since 2007 there have been three major incidents of farmed salmon mortality in BC that have been attributed to *Pseudochattonella* species, based solely on morphology of cells of the algal bloom species.

In 2012 HAMP received extra funding from DFO for the isolation, culture and genetic identification of harmful algae species from blooms. This allowed us to confirm the identification of a *Pseudochattonella* species from a fish-killing bloom in Quatsino Sound in late September 2013.

A. Lincoln MacKenzie [Ed]. *Marine and Freshwater Harmful Algae 2014*. Proceedings of the 16th International Conference on Harmful. Algae. Cawthron Institute, Nelson, New Zealand and the International Society for the Study of Harmful Algae (ISSHA)

Material and Methods

Quatsino Sound is a deep fiord on the north-western side of Vancouver, BC (Fig. 1). Marine Harvest Canada presently has four aquaculture facilities in the western area of the sound growing Atlantic salmon (*Salmo salar*). Water sampling is usually done twice daily at salmon aquaculture sites during the plankton season (March to October), using either a Niskin bottle, LaMotte sampler, or homemade sampling bottle. Discrete water samples are taken from 1-m, 5-m, and 10-m depths and examined live and / or preserved with Lugol's iodine solution, on Sedgewick Rafter slides, using a compound microscope with 40X to 400X magnification. Identification of harmful algae species in routine samples triggers more frequent sampling at the site. In addition, in Quatsino Sound, sentinel sites that are known to see harmful algae species earlier or at higher concentrations are sampled twice weekly.

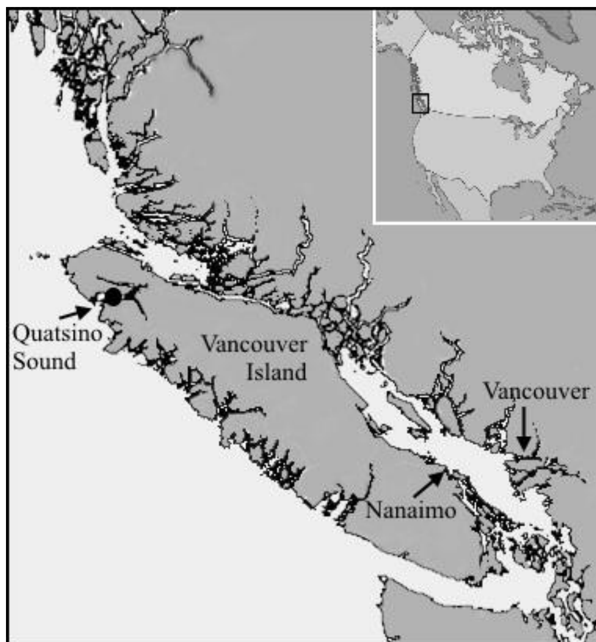

Fig. 1. Map of sampling area.

Dependent on the levels of harmful species seen, mitigation procedures are put into operation at the farm site; these include witholding feed, dropping barrier curtains around the pens, and diffusing air at depth to upwell deeper water into the pen system.

Environmental data, including water temperature, salinity, dissolved oxygen, and turbidity, are also collected daily at the farm site.

Live water samples collected from the *Pseudochattonella* bloom in Quatsino were taken to the DFO Pacific Biological Station algal lab facility in Nanaimo. Cells were isolated from bloom samples by serial dilution in 24-well plates (Guillard 1995) and grown up in modified ESAW with added silicate (Harrison *et al.* 1980).

A 30 mL sample of the established culture was centrifuged at 4° C at 4500 g for 10 minutes and the supernatent removed. The resulting cell pellet was stored at -80° C until processed. DNA was isolated from the sample using DNEasy extraction kit (Qiagen). Two times the recommended volume of Buffer ATL and Proteinase K was used in the lysis step, and the manufacturers directions were followed for the remainder of the protocol.

Real-time PCR was performed on a 96.96 dynamic array on the Fluidigm BioMark, according to standard Fluidigm protocols (Fluidigm Corporation, CA, USA) and as described in Miller *et al.* (2014). 10 ng/uL of extracted DNA was used as the starting material concentration. The real-time Taqman *Pseudochattonella* species primers and probe were as designed by Bowers *et al.* (2006).

The data was analysed with Real-Time PCR Analysis Software (Fluidigm Corporation, CA, USA).

Results and Discussion

Early September 2013 was unusually hot and dry on northern Vancouver Island, but near the fall equinox weather conditions started to deteriorate, with rain and wind increasing surface salinity and causing a turnover of the water column in Quatsino Sound (Fig. 2).

From September 26, fish behaviour at the Monday Rock farm site during the ebb tide suggested that there was an irritant present in the water, likely a harmful algae species. Quatsino Sound is an area that is frequently affected by HABs, and therefore has farm personnel that are highly trained in phytoplankton sampling, identification, and mitigation. The company plankton monitoring technician sampled extensively at farm and sentinel sites at this time, but only very low levels of known HAB species were seen.

On the night of September 27 there was another storm event in the area, with very strong wind and heavy rain. On the morning of September 28, Monday Rock farm personnel saw increasing fish distress at the turn to ebb tide at 1030h; an hour later visibility was very low (~0.5 m). Water samples were taken immediately, and showed high levels of small (~5 µm) flagellated cells of *Pseudochattonella* species; cell counts at 1130h were estimated to be 1000 cells mL^{-1} in the 1-m

sample. By 1430h these cells were visibly transforming into larger (~40 µm) oval to oblong cells, some of which became non-motile, others were still flagellated and motile (Fig. 3). Cell counts at 1445h on September 28 reached the maximum of 8000 cells mL^{-1} at 1-m depth. At this time extreme distress in the farmed salmon was evident, with a large mortality event estimated.

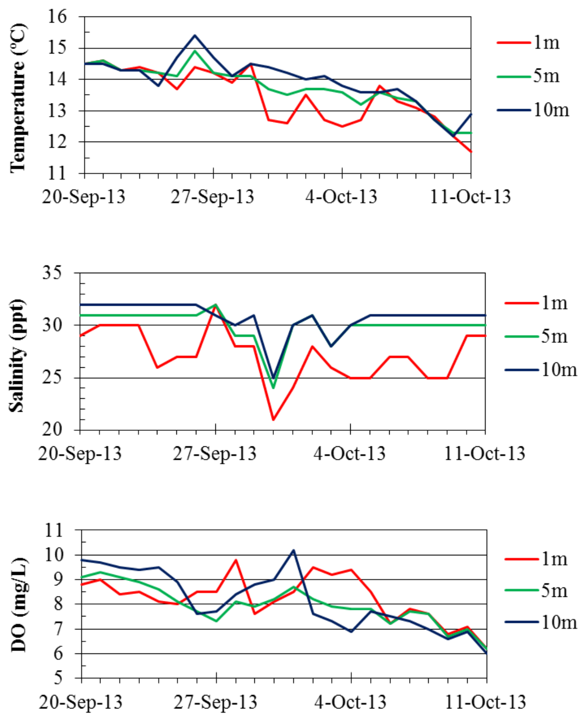

Fig. 2. Environmental conditions at Monday Rock farm site, Quatsino Sound, before and during *Pseudochattonella* sp. bloom, September – October 2013.

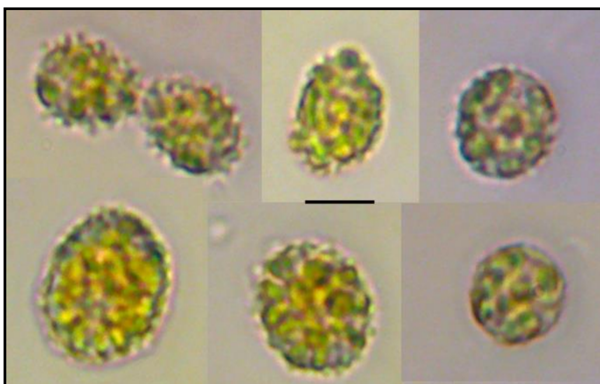

Fig. 3. Live *Pseudochattonella* sp. cells from Monday Rock samples. Scale bar = 10 µm.

This mortality event was short-lived however. In the days following, cell concentrations reduced rapidly (Fig. 4), and although there were subsequent increases in *Pseudochattonella* sp., by the evening of September 29 fish behaviour had returned to normal.

Water temperature at the beginning of the *Pseudochattonella* bloom was similar at the three sampling depths (13.9° – 14.1° C), with salinity increasing from 28 at 1-m to 30 at 10-m. Dissolved oxygen levels were high (Fig. 2).

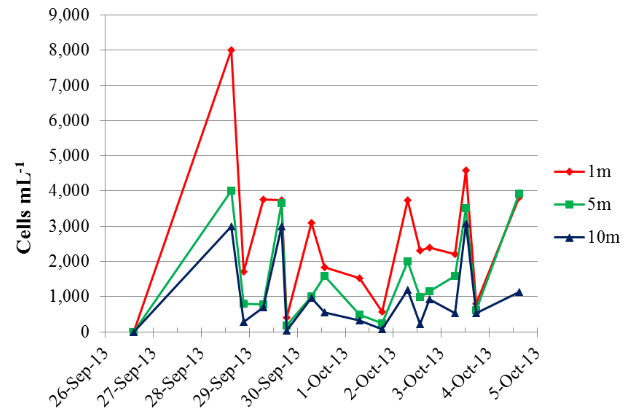

Fig. 4. *Pseudochattonella* sp. cell counts at Monday Rock farm site, September 26 – October 5, 2013.

Fresh Atlantic salmon killed by the bloom were examined after removal from the pens. Gross pathology showed extreme gill damage, with eroded lamellae and mucus production (Fig. 5). Histological examination of mortalities post-bloom by company fish health personnel revealed gill lamellar hyperplasia and fusion.

Fig. 5. Gross gill pathology of Atlantic salmon (*Salmo salar*) exposed to *Pseudochattonella* sp. bloom. Quatsino Sound, September 30, 2013.

Pseudochattonella species life cycles have been shown to have several stages, including small cells, motile and non-motile stages, and large multinucleate cells (Jacobsen *et al.* 2012; Chang *et al.* 2014). *Pseudochattonella* cf. *verruculosa*

from New Zealand has ”massive” plasmodium-like aggregates that produce many small swimming ”daughter” cells, which fuse to form larger cells. Chang *et al.* (2014) suggest that these massive aggregates may act as resting cells. Release of the daughter cells could therefore be a mechanism for initiating blooms; this gives a likely explanation for the initial observation of many very small flagellated *Pseudochattonella* sp. cells at Monday Rock. The rapid increase in *Pseudochattonella* cell counts in Monday Rock samples in the first few hours of the bloom are also thus most likely to be due to advection from the sediments rather than *in situ* growth.

Pseudochattonella species are very difficult to identify in live or Lugol's iodine preserved samples under the light microscope. In Quatsino Sound, because of previous *Pseudochattonella* blooms, farm personnel have some expertise at this, but identification based on morphology alone is tentative. Therefore an effort was made to isolate and culture cells from the bloom for molecular analysis.

Isolated *Pseudochattonella* sp. cultures from Monday Rock live samples were slowly acclimated to the Pacific Biological Station algal lab facility.

DNA from the cultured sample was successfully extracted and was amplified for *Pseudochattonella* sp. using real-time PCR. The sample was positive, with a cycle threshold (Ct) of 14 (equivalent to a Ct of ~22-24 with traditional rt-PCR platforms). This indicates a high concentration of the targeted *Pseudochattonella* sp. was present in the cultured sample.

The assay by Bowers *et al.* (2006) used in this study may not be able to accurately discriminate between the closely related *Pseudochattonella* species of *P. verruculosa* and *P. farcimen* (Dittami *et al.* 2013). As such, further examination is being conducted using high-throughput sequencing methods in order to verify the species identification.

Acknowledgements

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And always thanks to the HAMPsters: Jeanine and Tamara, for all their support.

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Paralytic Shellfish Monitoring in the Philippines - A Management Review

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Abstract

Paralytic shellfish poison (PSP) has been a perennial problem in the Philippines, with significant impacts on public health and the fisheries industry. To mitigate its impact, the Philippine government implemented a monitoring program that started in 1983. Current monitoring focuses on the toxin levels in shellfish and the mouse bioassay (MBA) is the official regulatory method. In 2010 a review, based on the analysis of a long term dataset, permitted the relaxation of the regulatory level from a very stringent level of >40 µg STX equivalent/100g to a level of >60 µg STX equivalent/100g which is just below the internationally recognized regulatory limit. Analysis results were incorporated in the issuance of regular advisories and bulletins. Capacity building for regional and local testing centers has been accomplished, however monitoring activities are restricted by the wide-spread archipelagic nature of the country, and limitations imposed by MBA. The Jasco High Performance Liquid Chromatography (HPLC) method is recognized as an alternative for toxin detection at times when test mice are not available. Immunoassay screening methods (the Abraxis ELISA and Jellett Rapid Test kits) for toxin analysis were evaluated and eventually adopted to address the limited supply of test mice in remote regions. This paper highlights the changes in monitoring strategies adapted by the BFAR over time.

Keywords: monitoring, management, paralytic shellfish poison

Introduction

Harmful algal blooms (HABs) are natural phenomena that may manifest in the environment causing fish kills or may pose a threat to human health. HABs also have economic effects due shellfish harvest closures and losses from unsold seafood products due to poison scares. At present, there is no means available to predict HAB occurrence (Okaichi, 2003). However, by conducting constant and regular monitoring in coastal waters, early detection of HABs is possible thus limiting its hazardous effect (Andersen *et. al*., 2001).

A number of Philippine coastal waters have been affected by toxic algal blooms that have caused contamination of shellfish with paralytic shellfish poison (PSP). Since the first recorded outbreak, PSP has been an important concern of the Philippines (Hermes and Villoso, 1983; Bajarias *et.al.,* 2006). To mitigate the negative impacts of PSP, the Bureau of Fisheries and Aquatic Resources (BFAR) implements a monitoring program to support management strategies (Gonzales, 1989a). Regular monitoring and surveillance is conducted to reduce the risk of ingestion of contaminated shellfish that could affect human health (Bajarias and Arcamo, 2005; Relox, J. *et.al*., 2009).

The first outbreak of PSP in the Philippines occurred in 1983 with *Pyrodinium bahamese* var. *compressum* (Pbc) as the causative organism (Gonzales, 1989b). Since then, a number of areas that BFAR is continuously monitoring, have been affected by Pbc. A number of cases involving fatalities have been recorded, the highest of which were in the late 1980s from the first reported outbreak and another peak in early 1990s (Fig. 1). There were no cases or fatalities recorded in 2000 and 2001. Moreover, there was an observed reduction in cases and fatalities in the 2000s compared to those noted in the 1980s and 1990s.
The core of the monitoring program is the shellfish toxicity analysis and the primary method

is mouse bioassay (MBA). The results of the toxicity analysis is the basis of regular shellfish bulletins and advisories.

The Monitoring Program

Management Authority

The PSP monitoring program started with a multi-sectoral task force composed of various government agencies which was characterized by a top-down and centralized approach from 1983 (Bajarias, 2002). Under this approach, closure and opening of shellfish harvesting areas was done through the issuance of signed joint statements from heads of two departments namely the Department of Agriculture and Department of Health (NRTTF & IACEH, 1989).

But such an approach was prone to delays in the issuance of the official notifications due to inter-office indorsements of papers and posed a number of difficulties in a country of an archipelagic nature.

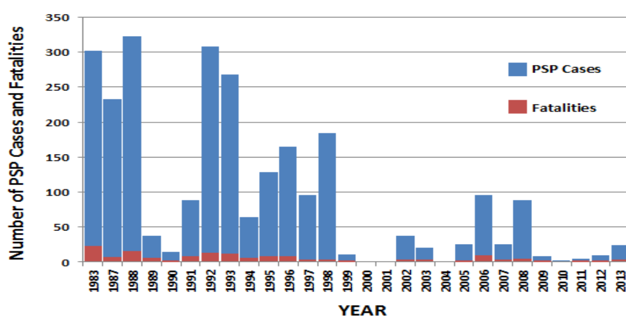

Fig. 1. Reported PSP cases in the Philippines

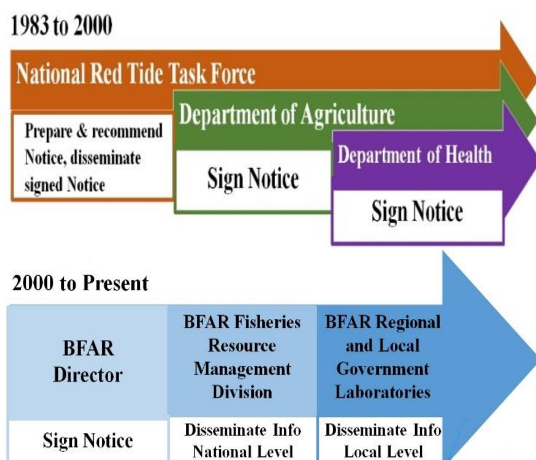

Fig. 2. Process flow showing the difference of the old and new decision and information flow

A review of existing regulations was conducted to streamline the process. As a result of the review and a series of consultations conducted thereafter, BFAR, the agency responsible for monitoring and ensuring sea food safety, took on the sole responsibility of monitoring and issuing of regular and timely updates in 2000 (Fig. 2). The BFAR partnered with its regional offices and local government units to cover the vast areas with a history of PSP. To ensure the competence of regional and local marine toxin testing centers, legislation was passed through Fishery Administrative Orders (FAOs) which dealt with monitoring procedures and accreditation of marine toxin testing centers by the BFAR central laboratory as the reference laboratory.

PSP Regulatory Limit

The Philippine regulatory criterion for PSP is more conservative than the international level of 80 µg STX equivalents/100g shellfish meat. The regulatory limit was initially set at 40µg STX equivalent/100g shellfish meat. At this level, observations showed a growing number of areas that were consistently placed under a shellfish harvesting ban. This prompted a review by the BFAR of its long term record of measured toxicity in shellfish against reported cases and fatalities in 2000. The result of this review showed that during the early part of the monitoring periods, toxicity in shellfish meat analyzed was not representative of the actual toxicity at the time of intoxication cases. Moreover, historical records showed that PSP cases were not observed at levels below 60 µg/100g and were validated by subsequent analysis records from 2000 onwards. Hence, this value was adapted as the regulatory limit through a Fishery Administrative Order that took effect since 2010.

Analytical Methods

The BFAR monitoring program is based on measurements of shellfish toxin concentrations for regulatory closures of shellfish harvest areas through the mouse bioassay (MBA) method for toxin quantification. However, this method sometimes suffers from unreliable supplies of test mice colonies and severe logistical hurdles in obtaining samples from remote areas that often impede timely monitoring assessments. Therefore, BFAR resorted to the use of alternative techniques such as the Jasco High Performance Liquid Chromatography (HPLC) method using prechromatographic oxidation and liquid chromatography with fluorescence detection (Lawrence *et. al.* 2005). Likewise, the possibility

of using other toxin detection methods such as enzyme-linked immunosorbent assay ELISA by Abraxis (product number 52255B) and Jellett Rapid Test (JRT) for PSP (Batch 40000-25Feb08) were explored. The U.S. Interstate Shellfish Sanitation Conference and U.S. Food and Drug Administration have recognized the Abraxis ELISA (ISSC, 2011) and JRT test kit (Van Dolah *et. al.,* 2012) as screening methods in a limited capacity.

Prior to their application, ELISA and JRT were tested on the various shellfish in the country (Arcamo, S.V.R. *et. al.* 2012). MBA results were used as reference. Comparison of MBA and JRT (Fig. 3) shows that the JRT gives positive results at critical MBA toxicity levels. In addition, positive JRT results were detected when MBA result were negative. These observations suggested that JRT can be used as early warning tool for possible shellfish contamination since JRT positive results are subject to MBA confirmatory test.

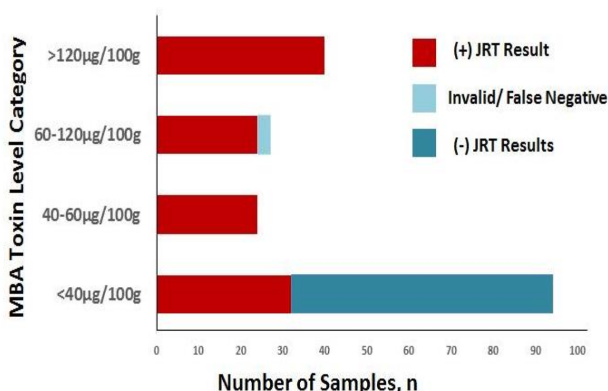

Fig. 3. Comparison of MBA with JRT results.

Comparison between MBA and ELISA (Fig. 4) shows that values obtained using ELISA were greater than MBA as indicated by an r^2 value of 0.5255. With these results, ELISA can be used

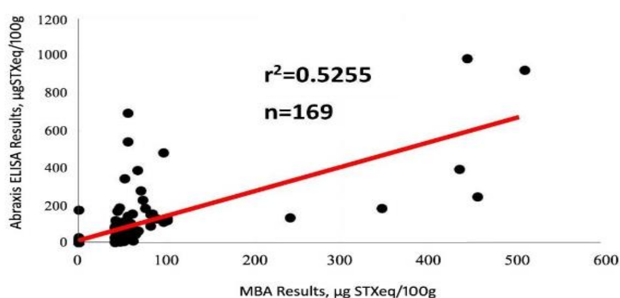

Fig. 4. Comparison of MBA with ELISA results.

for screening. A critical value was set and those which exceeded the value is subject to MBA confirmatory test. At the start of 2014, the BFAR Central Office distributed rapid test kits to its regional and local partner laboratories. Toxin analyses were done on site using these kits. Only the samples that tested positive were sent to the BFAR-Fisheries Resource Management Division (FRMD) Marine Biotoxin Laboratory for confirmatory tests. Confirmatory tests are done using MBA or HPLC. With this approach, the central laboratory is able to address the high volume of samples from regions and the usage of test mice is reduced.

Discussion

PSP impacts human well-being and the economy of the fisheries sector. The streamlining in PSP monitoring and management has facilitated better monitoring and surveillance to reduce the negative impacts and improve forecasting to allow timely information exchange to protect the shellfish industry and the consumers.

Relaxing the stringent national regulatory standard eliminated the immediate imposition of shellfish ban when PSP was detected. Seafood safety is still guaranteed under the current national regulatory standard since it is within the internationally recognized standard. In addition, there is a growing number of regional and local monitoring centers that are working collaboratively with the BFAR central office to cover a wider area of the country’s coastal waters. The staff manning regional and local laboratories were trained to measure shellfish toxin in their respective areas such that toxicity information is readily available, and regulatory and management actions can be easily implemented in a timely manner.

New technologies and advancement in toxin detection facilitated PSP monitoring. While the MBA is the official regulatory method, the test mice are available only in Manila. The MBA is labor intensive and the supply of test mice of the appropriate weight may not always be available. The use of HPLC in the central laboratory as an alternative method and the introduction of the new screening technologies to remote local areas led to a more pro-active approach in toxin detection and monitoring.

In summary, the evolution of the PSP monitoring system in the Philippines involved changes to several key aspects. The first was the change in authority to streamline decision making process and the flow of information which is critical to the enforcement of management strategies. Second was implementation of a less strict regulatory limit that enabled trading of shellfish that may be contaminated with very low levels of PSP but is still safe for consumption. Third was the active involvement of regional and local laboratories in sample collection and analysis aided by emerging new techniques in PSP toxin screening and detection. Finally and most importantly was the incorporation of these changes into Fisheries Administrative Orders which gives the legal basis for the implementation of the monitoring program. The challenge that BFAR now faces is to maintain its current monitoring approach which is possible through timely and proper planning.

Acknowledgements

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Monitoring of algal blooms along the southwest coast of India during 2008 to 2012

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Abstract

Regular surveillance was conducted along the coast of Kerala in Southwest India during the period 2008 to 2012, as part of the harmful algal monitoring programme of the Ministry of Earth Sciences, Government of India. Four major bloom events were recorded during the study period. A bloom of *Protoperidinium* sp. was observed off Mangalore during an interdisciplinary cruise on board FORV Sagar Sampada. A mono-specific bloom of *Prymnesium parvum* was observed off Azheekode during the monsoon period (August 2009) and a bloom of the centric diatom *Proboscia alata* was observed from 10th to 12th October 2009 along the coastal area near Bekalam. A massive bloom of the marine raphidophyte, *Chattonella marina* was observed in the coastal sea off Mahe from 27th October to 1st November 2011. Detailed investigations on the taxonomy, abundance and ecology of these microalgae were undertaken to understand the bloom dynamics.

Keywords: Harmful Algal Blooms, *Protoperidinium*, *Prymnesium parvum*, *Proboscia alata*, *Chattonella marina.*

Introduction

Algal blooms are quite common in Indian waters. Periodic blooms of *Noctiluca scintilans*, *Trichodesmium erythraeum* and *Rhizosolenia sp*. have been reported earlier (Bhat and Matondkar, 2004) whilst *Trichodesmium* and *Noctiluca* are the dominant genera which produce blooms (Naqvi *et al*., 1998; Eashwar *et al*., 2001; Dharani *et al*., 2004; Sarangi *et al*., 2004; Satish *et al*., 2005; Mohanty *et al*., 2007); a bloom of a holococcolithophore (Ramaiah *et al.* 2005) and *Coscinodiscus* (Padmakumar *et al*., 2007) have also been observed. Monitoring and surveillance of planktonic algal blooms along the coast of Kerala in southwestern India has been a part of the Harmful Algal Blooms project funded by the Centre for Marine Living Resources & Ecology under the Ministry of Earth Sciences, Government of India. Seasonal samplings were carried out along the southwest coast of India during 2008-12 (Fig. 1.) Four algal blooms were recorded during this period.

Material and methods

Microalgal samples were collected using a 20 micrometer mesh plankton net and preserved with Lugol's iodine solution. Identification of algae was made on the basis of standard taxonomic keys (Tomas, 1997). Hydrographic variables such as temperature, salinity, pH and dissolved oxygen (DO) were measured *in situ* using standard instruments. Chlorophyll *a* (Chl *a*) nutrients (nitrite, nitrate, phosphate and silicate were analysed as per Strickland and Parsons (1972).

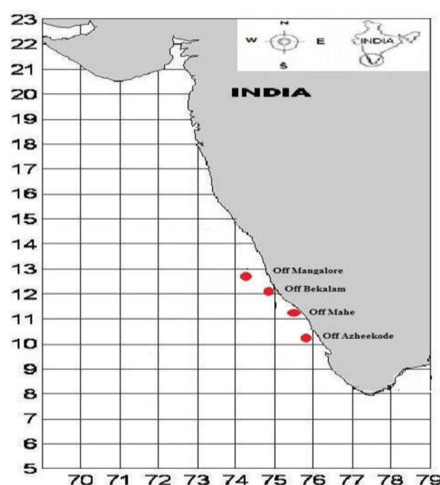

Fig.1. Map showing the locations where blooms were observed

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Results and discussion

Protoperidinium sp. bloom

This *Protoperidinium* dominated bloom was observed on 8th October, 2008 off Mangalore ($12^0$51.83'N & $74^0$20.00'E) during the multidisciplinary scientific expedition of FORV *Sagar Sampada* (Cruise#258). Visible discolouration of the water (pale red) was witnessed and the bloom was spread over an area of about 3.2 nautical miles in circumference. This was not a monospecific bloom; however, the dominant species belonged to the genus *Protoperidinium*. The total standing crop measured was 5×10^8 cells L^{-1}. Dinoflagellate species such as *Protoperidinium oceanicum, Ceratium furca, C. fusus, C. trichoceros* and *Prorocentrum gracile* also contributed significantly to the standing crop. Temperature, salinity and pH of the sampling site were 29^0C, 34.64 and 8.34 respectively. Dissolved oxygen concentration was moderate (6.5 mg L^{-1}). Nitrate concentration was below the detectable range while phosphate and silicate was in lower concentrations (0.245 and 1.68 µmol L^{-1}, respectively).

Prymnesium parvum bloom

During the summer monsoon of 2009, a mono-specific bloom of *Prymnesium parvum* (N. Carter) was observed off Azheekode (Lat. 10° 11' 02" N; Long. 76° 09'22"E), recorded for the first time from Indian waters. The colour of the water surface had turned pale brown and it extended around 8-10 nautical miles from the coast. However, there was neither foam production nor fish mortality during the bloom event. The bloom lasted only one day as heavy rain dissipated the cells. At the time of observation, the cell density of the mono-specific standing crop of *P. parvum* was 8×10^7 cells.L^{-1}. The chlorophyll *a* concentration was 13.5 µg.L^{-1}. This was high when compared to the concentrations observed at this station during the same season in 2008 and 2010, of 3.8 µg.L^{-1} and 2.6 µg.L^{-1} respectively. Chlorophyll *c* and carotenoid concentrations were 3.4 µg.L^{-1} and 1.9 µg.L^{-1} at the time of the bloom, which were also comparatively higher to those observed at the station during the summer monsoons of 2008 and 2010.

The concentration of nitrate (5.6 µmol.L^{-1}), and dissolved oxygen (1.41 mg.L^{-1}) from the site during the bloom were lower than compared to those obtained in the years immediately before and after the bloom. The phosphate concentration was 1.9 µmol.L^{-1} during the bloom, which was higher than the 0.36 µmol.L^{-1}recorded during the same time in 2008, 62 µmol.L^{-1} of silicate was recorded during the bloom. Net primary production was high (3.5 gC.m^{-3}. day^{-1}) when compared with the years immediately preceding and succeeding the bloom event.

Proboscia alata bloom

The third bloom event recorded during this period was that of the centric diatom *Proboscia alata* (Brightwell) Sandstrom (formerly *Rhizosolenia alata*) (Fig. 2 & 3). The bloom was located off the coast of Bekal (12° 38'02" N & 75° 04' 31" E) over an area of around 3 nautical miles from 10th to 13th October 2009, during the early post-monsoon (Anit *et al*., 2014). The water surface had a pale brown discolouration, however no fish mortality or foam production was observed. For comparison sampling was performed from two reference stations along the same latitude, one before and one after the bloom site. They were off Thykadapuram (12°22' 84"N & 75° 10'94"E) and off Puthur (12°55' 18"N & 74° 95' 18"E).

The total standing crop on 10th October comprised only *P.alata*, with 8×10^4 cells.L^{-1} which, by 12th October decreased to 2.8×10^3 cells.L^{-1}, as a few other diatoms and dinoflagellates appeared. During these days, both the reference stations showed a predominance of diatoms. Over the 3 days, there was a reduction in the concentrations of chlorophyll *a* (10.8 µg.$L^{-1}$to 6.5 µg.L^{-1}), chlorophyll *c* (4.6 µg.L^{-1} to 4.0 µg.L^{-1}), and carotenoid values (2.4 µg.$L^{-1}$to 2.0 µg.L^{-1}). Chlorophyll *b* concentrations were also low. In comparison, the average concentrations of chlorophyll *a* (4.3 µg.L^{-1}), chlorophyll *c* (2.3 µg.L^{-1}) and carotenoids (1.1 µg.L^{-1}) were lower at the two reference stations.

The sea surface temperature at the bloom site ranged between 27^0C and 28^0C over the 3 days, while the salinity was stable at 35. The pH reduced gradually from 8.4 to 8.2, which was still higher in comparison to the pH from the two reference stations (7.6 and 7.8 respectively).
Similar reduction in the concentrations of silicate (38.3 µmol.$L^{-1}$to 14.2 µmol.L^{-1}), nitrate (2.1 µmol.L^{-1} to1.4 µmol.L^{-1}), phosphate (1.4 µmol.L^{-1} to 1.20 µmol.L^{-1}), dissolved oxygen (5.4 mg.L^{-1} to 4.1 mg.L^{-1}) and primary production (1.9 gC.m^{-3}.day^{-1} to 1.05 gC.m^{-3}.day^{-1}) was observed over the 3 days of the bloom. Concentration of nitrite was

below the detectable range. In comparison, the concentrations of silicate at the 2 reference stations were lower (1.9 µmol.L^{-1} and 2.5 µmol.L^{-1} respectively) than the bloom site. Phosphate was also found to be very low. However, nitrate values at these stations were comparatively higher (3.03 µmol.L^{-1}and 2.7 µmolL^{-1}respectively). The dissolved oxygen values (6.3 mg.L^{-1} and 5.2 mg.L^{-1}) and net primary production (0.76 gC.m^{-3}.day^{-1} and 1.41 gC.m^{-3}.day^{-1}) varied slightly in comparison.

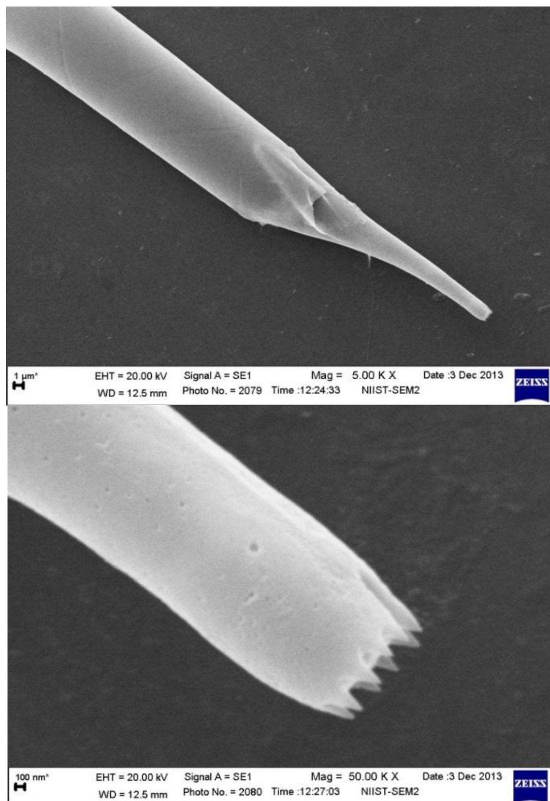

Fig. 2 & 3. Scanning Electron Micrographs of *Proboscia alata* (Brightwell) Sandstrom.

Chattonella marina bloom

A bloom of marine raphidophyte *Chattonella marina* (Subramanyan) Y.Hara *et* Chihara was the fourth event during this period which occurred from October 27th to November 1st 2011 off the coast of Mahe (Lat.11° 42'18"N, Long. 75° 32' 36"E) in north Kerala (Sanilkumar *et al.*, 2012). The conspicuous brown discolouration of surface water extended up to about two kilometres inside the Mahe (Mayyazhi) estuary during high tide and about 1 km towards the north and south of the bar-mouth. The gills of fishes were choked with the cells of *C. marina*. (Fig 4). The bloom was mono specific on the first day of the investigation (27th October) with the cell abundance of 4.5×10^6 cells.L^{-1}. This reduced gradually over the next 5 days to 3.8×10^5 cells.L^{-1}. A similar reduction in the concentrations of chlorophyll *a* (10.9 µg.L^{-1} to 6.7 µg.L^{-1}), chlorophyll *b* (0.7 µg.L^{-1} to 0.5 µg.L^{-1}), chlorophyll *c* (2.4 µg.L^{-1} to 1.8 µg.L^{-1}), and carotenoids (3.2 µg.L^{-1} to 2.3 µg.L^{-1}) was also observed. The sea surface temperature varied from 25°C to 27°C, while the salinity reduced from 30 on the first day of the bloom to 28 on the final day. pH varied between 7.9 and 7.7.

There was observable decrease in the concentrations of nitrate (12.5 µmol.L^{-1} to 7.0 µmol.L^{-1}), silicate (3.5 µmol.L^{-1} to 2.4 µmol.L^{-1}), and phosphate (0.6 µmol.L^{-1} to 0.5 µmol.L^{-1}), while the concentration of nitrite was below the range of detection. Dissolved oxygen concentration varied from 5.2 mg.L^{-1} to 4.4 mg.L^{-1} whereas net primary production ranged from 4.2 gC.m^{-3}.day^{-1} to 1.2 gC.m^{-3}.day-1 during the bloom event. Even though, *Chattonella marina* is capable of producing ichthyotoxins, to exert the toxic effect a minimum cell concentration is essential. The present HAB event was not a prolonged one and not much faunal mortality observed. Hence detailed investigations on ichthyotoxins, reactive oxygen radicals or free fatty acids produced by *C. marina* were not carried out.

Fig. 4.The gills of Mullet fish being choked with *C. marina* cells.

Conclusions

It could be inferred that these algal bloom events were naturally driven by physical forcing such as influence of monsoon, riverine discharge and seasonal upwelling, which result in variations in temperature, salinity, irradiance, water stability, and nutrient-enrichment (eutrophication). The optimum conditions for blooming vary from species to species. Occurrence of the *Chattonella*

marina bloom, was attributed to the presence of higher concentrations of nitrate, since N: P ratio was found to be high during the initial stage of the bloom. The optimum temperature for this bloom was found to be around 25°C.The occurrence of the *Proboscia alata* bloom was attributed to the monsoons, which coupled with upwelling, led to high nutrient conditions especially of silicate which imparted a high Si:N ratio that played a significant role in the bloom initiation. Even though there was no specific environmental factor that could be attributed to the *Prymnesium parvum* bloom, the eutrophic condition and optimum physical conditions could have favored the bloom event. The bloom was non-toxic in nature which might have been due to the availability of sufficient nutrients, nitrogen and phosphorus.

It can be concluded that the algal blooms were not triggered by a single factor but a combination of species specific, physical-chemical, geographical and biological factors played important roles in their development.

Acknowledgements

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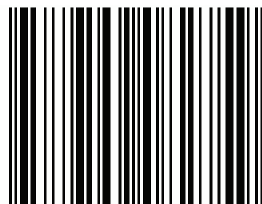
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